THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CAREDX, INC. and THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY)
)
)
)

Plaintiffs,)
)
)
v.)　C.A. No. 19-cv-1804-CFC-CJB
)
EUROFINS VIRACOR, INC.,)
)
Defendant.)
)
)
)

JOINT APPENDIX IN SUPPORT OF CLAIM CONSTRUCTION BRIEF

Dated: April 16, 2021

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A0009-94	Declaration of Dr. Steven Weisbord In Support of CareDx's Reply Claim Construction Brief and supporting Appendices
A0095-98	Declaration of John F. Beausang **(Intrinsic Evidence)**
A0099-102	PCT/US2010/055064 – May 4, 2012 Claims **(Intrinsic Evidence)**
A0103-105	U.S. Patent Application No. 13/508,318 – August 23, 2013 Applicant Initiated Interview Summary **(Intrinsic Evidence)**
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A0131-145	Excerpt of Deposition Transcript of Uwe Christians, M.D., Ph.D. taken November 9, 2020, C.A. No. 19-cv-662 (D. Del.)
A0146-201	Excerpt of Deposition Transcript of Uwe Christians, M.D., Ph.D. taken March 29, 2021, C.A. Nos. 19-cv-567, 19-1804 (D. Del.)
A0202-204	U.S. Patent Application No. 13/508,318 – March 21, 2013 Claims **(Intrinsic Evidence)**
A0205-214	U.S. Patent Application No. 13/508,318 – May 10, 2013 Non-Final Rejection **(Intrinsic Evidence)**

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A0235-242	Wu, et. al. "A Schema For Histologic Grading of Small Intestine Allograft Acute Rejection", Transplantation, 75:8 (April 2003) 1241-1248.
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A0281-291	Pollack, et. al. "Detection and Imaging of Cardiac Allograft Vasculopathy", JACC: Cardiovascular Imaging 6:5 (May 2013) 613-623.
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A0307-310	G. Singh, "Determination of Cutoff Score for a Diagnostic Test", Internet J. of Laboratory Medicine 2:1 (2006)
A0311-317	Excerpt of the Rebuttal Expert Report of Uwe Christians, M.D., Ph.D. dated October 16, 2020, C.A. No. 19-662 (D. Del)
A0318-999	INTENTIONALLY OMITTED
A1000-1005	U.S. Patent Application No. 13/508,318 – 1/14/2014 Response to Office Action (claim 36 before amendment in Notice of Allowability) **(Intrinsic Evidence)**
A1006-1013	U.S. Patent Application No. 13/508,318 – Notice of Allowability **(Intrinsic Evidence)**
A1014-1017	U.S. Patent Application No. 13/508,318 – Beausang Declaration **(Intrinsic Evidence)**
A1018-1023	Snyder et al., Universal noninvasive detection of solid organ transplant rejection, *PNAS* 108:15 (2011)
A1024-1028	January 27, 2021 Email from Anna Dwyer
A1029-1037	U.S. Patent Application No. 13/508,318 – Office Action Summary **(Intrinsic Evidence)**
A1038-1048	Ross et al., The Role of Echocardiography in the Follow-Up of Orthotopic Heart Transplant Patients, *Cardiol. Rev.* 4:6 (1996)

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A1049-1100	Declaration of Uwe Christians, M.D., Ph.D. In Support of Defendant Eurofins Viracor, Inc.'s Answering Claim Construction Brief
A1101-1107	Excerpts of Deposition Transcript of Dr. Brian Van Ness, Ph.D.
A1108-1118	Excerpts of Declaration of Dr. Brian Van Ness, Ph.D.
A1119-1124	INTENTIONALLY OMITTED
A1125-1142	Bloom et al., Cell-Free DNA and Active Rejection in Kidney Allografts, *J Am Soc Nephrol* 28 (2017)
A1143-1147	Bossuyt et al., Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative, *BMJ* 326 (2003)
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A1229-1265	Zweig et al., Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics (ROC) Plots; Approved Guideline, *NCCLS* 15:19 (1995)
A1266-1367	Curriculum Vitae of Uwe Christians, M.D., Ph.D.
A1368-1390	Excerpts of Deposition Transcript of Steven Weisbord, M.D., Friday, April 9, 2021
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A1397-1400	Excerpts of Deposition Transcript of Uwe Christians, M.D., Ph.D., Monday, March 29, 2021
A1401-1404	Sharples et. al, Diagnostic Accuracy of Coronary Angiography and Risk Factors for Post-Heart Transplant Cardiac Allograft Vasculopathy, *Transplantation*, 76(4):679-682 (2003)
A1405-1410	Kobashigawa et al., Multicenter Intravascular Ultrasound Validation Study Among Heart Transplant Recipients, *Journal of the American College of Cardiology*, 45(9) (2005) **(Intrinsic Evidence)**
A1411-1429	Miniati, Douglas N. & Robbins, Robert C., Heart Transplantation: A Thirty-Year Perspective, *Annu. Rev. Med.*, 53:189-205 (2002)

APPENDIX

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Top Skills

Chemistry
Organic Chemistry
Intellectual Property

Amy Bunker

Research Scientist at AAPharmSyn
Ann Arbor

Experience

U.S. Patent & Trademark Office
Patent Examiner
July 2012 - Present (8 years 8 months)
Alexandria, VA

AAPharmaSyn
Research Scientist
November 2010 - July 2012 (1 year 9 months)

AAPharmaSyn (AAP) is a global chemistry contract research organization based in Ann Arbor, Michigan.

Cayman Chemical
Legal Counsel
2008 - February 2010 (2 years)

Brooks Kushman P.C.
Law Clerk
2007 - 2008 (1 year)

Pfizer Inc. (Ann Arbor)
Senior Scientist
2001 - 2007 (6 years)

Bristol-Myers Squibb
Research Scientist II
1997 - 2001 (4 years)

Parke-Davis Warner-Lambert
Senior Associate Scientist
1990 - 1997 (7 years)

Chemsyn Science Laboratories
Associate Scientist
1987 - 1990 (3 years)

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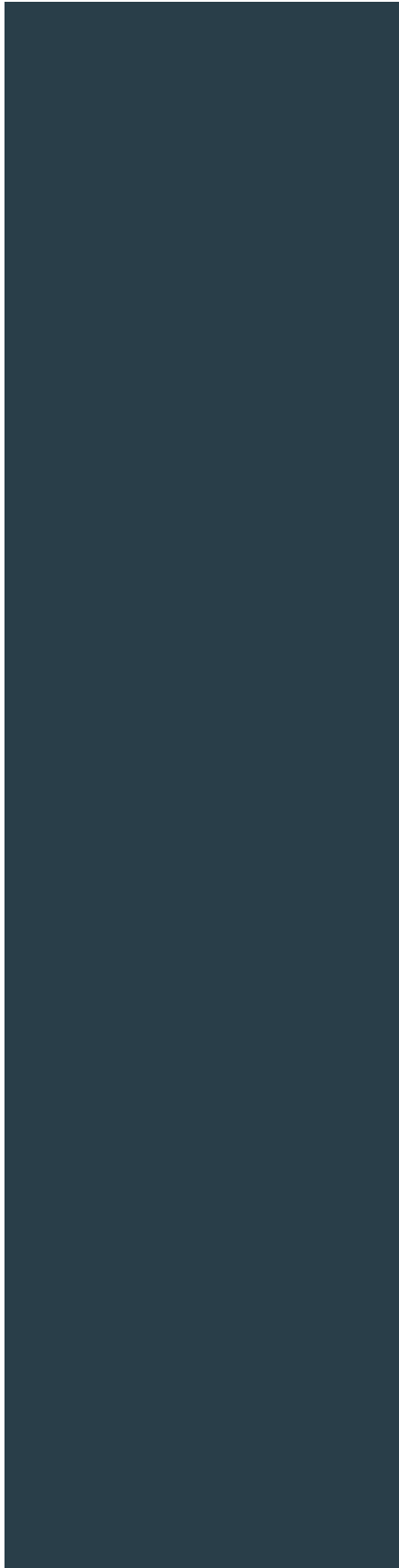
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JD, Law · (2003 - 2008)

Central Michigan University
MS, Chemistry · (1985 - 1987)

Michigan State University
BA, Chemistry · (1981 - 1985)

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Maria Leavitt

Primary Patent Examiner at USPTO
McLean

Experience

USPTO
Primary Patent Examiner
June 2005 - Present (15 years 9 months)
US Patent and Trademark Organization, Art Unit 1633

Biotechnology

National Institute of Health
Research Fellow
June 2003 - June 2005 (2 years 1 month)
National Cancer Institute

HIV-1 HIV-2 vaccine development

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Education

Eastern Virginia Medical School
PhD, Reproductive Endocrinology · (1993 - 1998)

Old Dominion University
Master Sciences, Endocrinology · (1988 - 1991)

Old Dominion University
Bachelor Art, Spanish Education · (1980 - 1984)

Universidad Complutense de Madrid
Licenciada Ciencias Biologicas, biologia general · (1975 - 1979)

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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CAREDX, INC., 　　　　　Plaintiff, 　　v. EUROFINS VIRACOR, INC., 　　　　　Defendant, and THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY 　　　　　Nominal Defendant.	))))))　C.A. No.: 1:19-cv-1804-CFC)))))))))))

EUROFINS VIRACOR, INC.'S INITIAL INVALIDITY CONTENTIONS

Pursuant to Paragraph 4(d) of the District of Delaware Default Standard for Discovery, Defendant Eurofins Viracor, Inc. ("Eurofins Viracor" or "Defendant"), hereby provides to Plaintiff CareDx, Inc. and Nominal Defendant The Board of Trustees of the Leland Stanford Junior University (collectively, "CareDx") the following initial invalidity contentions with respect to U.S. Patent No. 8,703,652 ("the '652 Patent"). CareDx has asserted claims 1-4, 6, 11-12, and 14-15 of the '652 Patent (the "Asserted Claims") against Eurofins Viracor.

Eurofins Viracor makes these Contentions based on its current knowledge, recognizing that investigations are continuing and discovery is ongoing. For example, CareDx has not yet produced any documents in response to Eurofins Viracor's requests for production, and depositions of the alleged inventors have not yet taken place. As such, Eurofins Viracor reserves the right to modify, amend, or otherwise supplement these Contentions in view of further

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<div align="center">**A0004**</div>

written description, however, does not describe any method with a sensitivity "greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)." The specification states that "[i]n some embodiments, the methods described herein have at least 56% sensitivity." *Id*. at 2:44-45. But the specification does not describe a single example, or any results, showing that the inventors had in fact obtained a sensitivity of "greater than 56%." To the extent the limitation is not inherent, it is not adequately described in the specification.

Furthermore, the claims are not limited to any particular transplant or organ. The specification, however, does not describe a representative number of species (i.e., specific transplants or organs) that are representative of the entire genus. Indeed, the sole working example (Example 2) does not even identify what transplant or organ was used. There is furthermore no description of how surveillance methods for cardiac allograft vasculopathy (CAV) would apply in the context of transplant rejection of other organs. Accordingly, the specification fails to provide adequate written description.

VIII.   CLAIMS 1-4, 6, 11-12, AND 14-15 ARE INDEFINITE UNDER 35 U.S.C. § 112

In the alternative, claims 1-4, 6, 11-12, and 14-15 are indefinite and are therefore invalid under 35 U.S.C. § 112. Independent claim 1 is directed to a "method for detecting transplant rejection, graft dysfunction, or organ failure . . . wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)." The phrase, when read in light of the specification and prosecution history, fails to inform a POSA about the scope of the claim with reasonable certainty.

The claim language itself does not provide reasonable certainty on the scope of the claim. The phrase "wherein sensitivity of the method is greater than 56%" is modified by the phrase

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"compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)," but it is not clear how that modification imparts any meaning to the entire phrase that is different than the meaning of "wherein sensitivity of the method is greater than 56%" alone. The claim language also refers generally to "current surveillance methods for cardiac allograft vasculopathy (CAV)," but is not limited to any specific methods. It is also not clear from the claim language whether the phrase "current surveillance methods" refers to then-current methods available at the time the application was filed, at the time of alleged infringement, or at the time of some other reference timepoint. The claim language does not make clear whether the claimed sensitivity refers to the sensitivity of a clinical diagnosis or, instead, the sensitivity of the sequencing instrument. Furthermore, the phrase refers to "methods for cardiac allograft vasculopathy," but the claim is not limited to a cardiac method, and therefore it is unclear to a POSA how to compare a method for kidney transplant, for example, against a method for CAV.

The specification also fails to provide reasonable certainty on the scope of the claim. For example, the specification states that "[c]urrent surveillance methods for CAV lack adequate sensitivity or require invasive procedures and the most commonly applied method, coronary angiography, lacks sensitivity." '652 Patent at 6:8-11. The cited reference, Kobashigawa, J. A., *et al.*, *J. Am Coll Cardiol*, 45, 1532-1537 (2005), does not provide any information on sensitivity of any methods. The specification also does not provide any working example in which a sensitivity was reported, much less a sensitivity compared against "current surveillance methods for cardiac allograft vasculopathy."

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Dated: January 14, 2021

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CERTIFICATE OF SERVICE

I hereby certify that on January 14, 2021, **EUROFINS VIRACOR, INC.'S INITIAL**

INVALIDITY CONTENTIONS, was served electronically on counsel for Plaintiff CareDx Inc.

and Nominal Defendant The Board of Trustees of the Leland Stanford Junior University.

Dated: January 14, 2021

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UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CAREDX, INC. and THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY Plaintiffs, v. EUROFINS VIRACOR, INC., Defendant,	C.A. No. 19-cv-1804-CFC-CJB

DECLARATION OF DR. STEVEN WEISBORD IN SUPPORT OF CAREDX'S REPLY CLAIM CONSTRUCTION BRIEF

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I, Dr. Steven Weisbord, hereby declare as follows:

I.   Introduction and Summary of Opinions

1.   My name is Dr. Steven Weisbord. I have been retained by Weil, Gotshal & Manges LLP, counsel for CareDx, Inc. and The Board of Trustees of the Leland Stanford Junior University (collectively, "Plaintiffs"). I understand that Plaintiffs have asserted that Defendant Eurofins Viracor, Inc. ("Viracor" or "Defendant") infringes claims 1-4, 6, 11-12, and 14-15 of U.S. Patent No. 8,703,652 (the "'652 Patent"). Counsel for Plaintiffs have asked me to provide opinions relating to the meaning of the phrase "wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)," which is recited in claim 1 of the '652 Patent. I have also been asked to respond to Defendant's expert, Dr. Uwe Christians' opinions that this claim term is indefinite. It is my opinion that the claim term "wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)" does not render the claim terms indefinite, and I disagree with Dr. Christians' opinions for the reasons set forth below.

2.   I have reviewed the '652 Patent claims and the intrinsic record, including the '652 Patent specification and prosecution history. In my opinion, a person of ordinary skill in the art ("POSA") at the time of the invention reading the

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'652 Patent claims in light of the intrinsic record would be able to determine the scope of the claims with reasonable certainty.

3. In particular, a POSA would understand that the "wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)" limitation requires a simple numerical comparison between the sensitivity of the claimed dd-cfDNA methods to the standard approach for surveilling CAV at the time of the invention, coronary angiography.

4. I have also reviewed the '607 Patent claims and the intrinsic record. In my opinion, a POSA at the time of the invention reading the '607 Patent claims in light of the intrinsic record would be able to determine the scope of the claims with reasonable certainty. Specifically, a POSA would readily understand the term "allele frequency greater than 1% of a population" recited in claim 2. Additionally, a POSA would understand that the "population" referred to in this claim refers to the relevant population for the claims, specifically, the population of individuals who could donate or receive a kidney transplant.

II. Qualifications

5. My qualifications are set forth in my *curriculum vitae* in A0057-92 (Appendix A). I currently hold several positions. First, I am a tenured Professor of Medicine and Clinical and Translational Science in the Renal-Electrolyte Division

of the University of Pittsburgh School of Medicine in Pittsburgh, PA. I have been a

faculty member at the University of Pittsburgh School Of Medicine since 2004.

Second, I am a Staff Physician in the Renal Section of the VA Pittsburgh Healthcare

System, also in Pittsburgh, PA. I have held this position since 2004. Third, I am a

Core Investigator of the Center for Health Equity Research and Promotion, a Center

of Innovation at the VA Pittsburgh Healthcare System. I have held this position since

2004. Finally, I am a Faculty Member at the Center for Critical Care Nephrology in

the Department of Critical Care Medicine of the University of Pittsburgh School of

Medicine. I have held this position since 2016.

6.   I earned a Bachelor's of Arts in Biology in 1990 from the University of

Rhode Island. I earned my MD from George Washington University in 1997. I next

served as a resident in internal medicine at the University Of Pittsburgh School Of

Medicine from 1997-2000. I became the Chief Resident in Internal Medicine at the

University of Pittsburgh in 2000, and then a Fellow in the Renal Electrolyte Division

in 2001. I earned my Masters of Science in Clinical Research from the University

of Pittsburgh in 2004.

7.   I am a licensed physician with a Board Certification in Nephrology. I

previously held a Board Certification in Internal Medicine from the years of 2000-

2010. I also hold a Clinical Nephropathology Certificate from the International

Society of Nephrology. I further belong to the American Society of Nephrology,

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where I have been a Fellow since 2005, and the National Kidney Foundation, where I have been a Fellow since 2011. I have been working with kidney transplant patients for nearly 20 years.

8.   I have received a number of awards for my work including the Pfizer American Society of Nephrology Fellow Abstract Award in 2002, the Department of Medicine Annual Research Day Post-Doctoral Fellow Research Award in 2003, the Pfizer American Society of Nephrology Fellow Abstract Award in 2003, a Division of Critical Care Medicine Institutional NRSA Awardee from 2001 to 2003, and the Raymond M. Rault Faculty Teaching Award in 2015. I was also the Bronze medal winner for the "Rookie of the Year Award in Excellence in Government" for my work as part of the VA Pittsburgh Healthcare System in 2005.

9.   Overall, I am the author or contributor on more than sixty-eight original refereed publications, fifty-eight reviews, editorials, and book chapters, and thirty published abstracts in the area of kidney disease, nephrology, and clinical studies. I have also been invited to speak at more than eighty seminars and lectureships.

10.   I have been involved in twenty-two funded research studies, including in the area of kidney transplantation, as either a Principal Investigator ("PI") or a Co-Investigator with total funding in excess of $35,000,000. I have also served as a Grant Reviewer for the National Institutes of Health ("NIH") – Renal and Urological Sciences Integrated Review Group (2008), NIH – Special Emphasis Panel/Scientific

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Review Group (2016), and the University of Pittsburgh Competitive Medical

Research Fund (2019). I have been a PI for more than twelve studies, including

prospective studies, retrospective studies, and clinical trials. My most recent work

as a PI was for a multi-center clinical trial of interventions for the prevention of acute

kidney injury that was published in the New England Journal of Medicine and as a

PI on a federally funded project investigating processes of care and outcomes among

kidney transplant recipients.

       11.   I also serve or have served as an Editor or on the Editorial Board of

multiple journals. I have served on the Editorial Board of the Clinical Journal of the

American Society of Nephrology and the Journal of the American Society of

Nephrology. I have held these positions since 2011 and 2017, respectively. I am an

Associate Editor for BMC Nephrology since 2015. I was a Guest Editor on the

Advances in Chronic Kidney Disease – Psychosocial and Quality of Life Issues in

Chronic Kidney Disease in 2007. Finally, I was an Editor for the UPMC Renal

Grand Rounds, UPMC Renal-Electrolyte Division. As part of my work for these

various publications, I have had to review and evaluate the design and

methodological robustness of countless clinical studies. Furthermore, I have served

as an invited reviewer for dozens of peer reviewed publications, including the New

England Journal of Medicine, where I was tasked with evaluating and assessing the

quality of hundreds of submitted studies.

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12.   Based on my experience, qualifications, and expertise, I am qualified as an expert in the field of kidney transplantation and associated treatments including monitoring of patients following transplantation and assessing the need for post-transplant procedures, the field of nephrology, and clinical studies and research.

III.   Compensation

13.   I am being compensated for my time at a rate ranging of $400 per hour for non-testimonial work and $750 per hour for testimony. My compensation is not dependent in any way upon the outcome of this proceeding.

IV.   Materials Considered

14.   Beyond having general and specific expertise from my educational background and work, I have also performed a careful and extensive review of documents and evidence specific to this litigation including all items cited herein and publicly available information. This list of materials I have reviewed is attached hereto as A0093-94 (Appendix B).

V.   Legal Background

15.   I am not a lawyer, but have been informed that definiteness is to be evaluated from the perspective of a POSA, that claims are to be read in light of the patent's specification and prosecution history, and that definiteness is to be measured as of the time of the patent application.

16.   I have been informed that a patent is invalid for indefiniteness if its claims, read in light of the specification delineating the patent, and the prosecution

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history, fail to inform, with reasonable certainty, those skilled in the art about the scope of the invention.

17.   I have been informed that some modicum of uncertainty may be tolerated, but the patent and prosecution history must disclose a single known approach or establish that, where multiple known approaches exist, a POSA would know which approach to select.

18.   I have been informed that if a POSA would choose an established method of measurement, that may be sufficient to defeat a claim of indefiniteness, even if that method is not set forth in *haec verba* in the patent itself.

VI.   The Person Of Ordinary Skill In The Art

19.   I understand that in determining the scope of patent claims, the claims are analyzed from the perspective of a person of ordinary skill in the art ("POSA") at the time of the invention.

20.   I have been advised that in determining the level of ordinary skill in the art, one should consider: (1) the levels of education and experience of persons working in the field; (2) the types of problems encountered in the field; and (3) the sophistication of the technology.

21.   In my opinion, a POSA as of November 6, 2009 would have had a Ph.D. in genetics, molecular biology, bioinformatics or a related field, and at least three years of research in an academic or industry setting, including at least two to three

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years of research experience in the field of human genomics. A POSA would have had knowledge of DNA sequencing, including next generation sequencing ("NGS") and related sequencing methods for grouping and comparing sequence reads and mapping sequence reads onto genomes, and methods for identifying genetic variants in a sample. A POSA would also have knowledge in computational approaches to quantitate DNA detected by the above techniques.

22.   I have reviewed the definition of a POSA offered by Viracor's expert, Dr. Uwe Christians. In his declaration, Dr. Christians states that "a POSA to which the '652 and '607 Patent claims are directed would have had a high level of education, such as a Ph.D. or M.D., with at least three years' experience in transplant medicine, transplant immunology, and medical diagnostics, or a similar field, including experience with transplant patient monitoring, transplant immunology and transplant injury. In determining the level of ordinary skill in the art, more education could compensate for less experience, and vice versa. A POSA would also have had several years of experience in the field of genomics, and/or would be one that could call upon or consult with someone with at least a bachelor's degree in a life or physical sciences discipline with applications in genomics, and several years' experience working in the field of genomics."

23.   While Dr. Christians' definition of a POSA differs from my own, my conclusions remain the same under either definition.

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VII. Opinions

A. Background On The '652 Patent

24. The technology that is the subject matter of the '652 Patent relates to improved, non-invasive tests for identifying organ injury, particularly the rejection of a transplanted organ using donor-derived cell-free DNA (dd-cfDNA).

25. A crucial factor in determining whether an organ transplant will succeed or fail is whether the transplant recipient's body accepts the donated organ. The transplant recipient's immune system poses a significant threat to the viability of the transplanted organ. Specifically, because a transplanted organ is a foreign substance, the transplant recipient's immune system will attack the transplanted organ. This is commonly referred to as organ rejection. Organ rejection can cause serious complications to the transplant recipient, and, in some cases, it can be deadly. While organ transplant recipients are prescribed immunosuppressive medication beginning at the time of transplantation, if a transplant recipient experiences organ rejection, his or her immunosuppressive treatment will be augmented and/or modified to further suppress the immune system and treat the rejection episode. While immunosuppressive medications dampen the body's immune response, which reduces the risk of organ rejection, they place the patient at higher risk of infectious complications.

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26.   Because the severity of complications from rejection increase with the severity of the rejection, and typically with the time it takes to diagnose and implement treatment, it is crucial to monitor the status of an organ transplant to detect potential organ rejection as early as possible.

27.   Before the inventions described in the '652 Patent, the status of an organ transplant with regard to injury/rejection was established or definitively diagnosed by performing a biopsy of the organ. While biopsies were very effective at, and are still the standard for, diagnosing organ rejection, they present risks to the patient. For one, a biopsy is an invasive procedure that requires needle extraction of organ tissue. Biopsies are also expensive and may cause the patient and the donated organ significant bleeding and, in some instances, organ failure. Some biopsies are also associated with patient discomfort and inconvenience. Standard non-biopsy surveillance methods available to a clinician commonly lacked sensitivity, specificity, and predictive value. As a consequence, other non-invasive methods were sought to identify organ rejection in its early stages, prior to the progression of the rejection and symptom onset. Due to the lack of effective and reliable methods to identify incipient organ rejection, many patients experienced irreversible organ dysfunction, organ failure, and even death by virtue of the lack of effective, non-invasive surveillance techniques for organ rejection.

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28.   Due to these shortcomings, there was a need prior to the invention for a more accurate non-invasive test for surveilling or determining the status of an organ transplant early on before more advanced rejection developed. The inventions claimed in the '652 Patent accomplish this need by measuring very small amounts of cell free DNA from the organ donor ("donor-derived cell-free DNA" or "dd-cfDNA") circulating in the transplant recipient's blood. According to the methods described in the '652 Patent, organ transplant status, including rejection, is determined by first drawing a blood sample from the transplant recipient containing a mix of donor and recipient cfDNA, and then analyzing that sample to determine the degree of dd-cfDNA in the patient's blood relative to the recipient's cfDNA. If the dd-cfDNA present in the transplant recipient's bloodstream increases or exceeds a certain threshold, that indicates that organ rejection may be occurring. The ability of these techniques to detect small increases in the amount of dd-cfDNA in the transplant recipient's blood before other markers of possible rejection are present increases the sensitivity of the surveillance test relative to other non-invasive approaches, and therefore allows a physician to detect organ rejection early on, implement treatment at an earlier stage of injury, and avoid some of the serious, adverse consequences described above.

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B.   The '652 Patent Specification

29.   The specification of the '652 Patent describes the challenges associated

with early detection of organ transplant rejection outlined above. For example, the

specification teaches that while monitoring transplant patients for transplant status

or outcome is difficult, expensive, and invasive, it is important to monitor transplant

status to detect changes in status and improve patient outcomes. D.I. 89-3 at 5:54-

64. As an example, the specification teaches that at the time of the invention,

patients who had received heart transplants experienced late mortality due to cardiac

allograft vasculopathy ("CAV"), which was the major cause of late graft failure and

death. *Id*. at 5:61-67. To avoid these outcomes, the specification explains the

importance of detecting CAV early and prior to the development of angiographically

apparent disease, graft dysfunction, or symptom onset. *Id*. at 6:1-8.

30.   The specification explains that coronary angiography was the standard

of care for monitoring patients who had received heart transplants for CAV. *Id*.

Indeed, according to the specification, coronary angiography was the most

commonly applied method for surveilling CAV. *Id*. at 6:8-11. Unfortunately,

coronary angiography was invasive and it lacked adequate sensitivity; it was not

sensitive enough to detect early onset CAV, which resulted in unacceptably high

rates of serious, adverse outcomes. *Id*. at 6:3-12.

31.   The specification describes another test that was available at the time of the invention for identification of CAV, intravascular ultrasound ("IVUS"). *Id.* at 6:12-15. Although IVUS was more sensitive than coronary angiography, IVUS was still invasive, costly, resource intensive, and associated with significant risk of morbidity and patient discomfort. *Id.* at 6:16-19. Consequently, the specification explains that there was a need to develop markers for early, non-invasive, safe, and cost-effective detection of CAV and decrease the cost of long-term management. *Id.* at 6:19-53. Indeed, the specification explains that the same difficulties and expenses associated with monitoring and detecting CAV in heart transplant recipients are experienced by patients receiving other types of transplants. *Id.* at 6:54-55.

32.   The '652 Patent thus describes and claims non-invasive methods for determining whether organ rejection is occurring, with sensitivities of greater than 56%. *See id.* at 2:44-45; 23:31-36. These innovative techniques represented a significant advance for the surveillance of organ status compared with the sensitivity of coronary angiography as a surveillance method for CAV described in the specification, which had a recognized sensitivity of only 43-44%.

C.   Background On Clinical Diagnostic Sensitivity

33.   I agree with Dr. Christian's description of how sensitivity is defined. Specifically, I agree that sensitivity is defined as the frequency of predicted positive diagnoses of a condition relative to the number of subjects known to have the

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condition.   A POSA reading the claims of the '652 Patent would therefore understand that the sensitivity of the claimed method simply refers to the frequency of predicted positive diagnoses of organ transplant rejection relative to the number of subjects known to have this condition.

34.   I also agree that to determine the sensitivity of a diagnostic assay, one would need to know the number of subjects who actually have the condition, and that a POSA would therefore compare a given diagnostic assay to a reference standard that is used to confirm the presence of a particular condition.

VIII.   The Sensitivity Term Is Not Indefinite

35.   Based on the plain language of the claims, a POSA would understand with reasonable certainty that the claims encompass assays with a sensitivity of greater than 56%, which was higher than the sensitivity of the standard method that was available and used for surveilling CAV at the time of the invention.

36.   Independent claim 1 of the '652 Patent sets forth the representative embodiment of the claimed dd-cfDNA surveillance methods:

> 1. A method for detecting transplant rejection, graft dysfunction, or organ failure, the method comprising:
>
> (a) providing a sample comprising cell-free nucleic acids from a subject who has received a transplant from a donor,
>
> (b) obtaining a genotype of donor-specific polymorphisms or a genotype of subject-specific polymorphisms, or obtaining both a genotype of donor-specific polymorphisms and subject-specific polymorphisms, to establish a polymorphism profile for detecting donor cell-

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free nucleic acids, wherein at least one single nucleotide polymorphism (SNP) is homozygous for the subject if the genotype comprises subject-specific polymorphisms comprising SNPs:

(c) multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile to detect donor cell-free nucleic acids and subject cell-free nucleic acids; and

(d) diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the donor cell-free nucleic acids based on the detection of the donor cell-free nucleic acids and subject cell-free nucleic acids by the multiplexed sequencing, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure, and *wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)*.

Id. at Claim 1.

37.   The steps of the claimed methods are clear from the plain language of the claims, particularly when read in light of the disclosures in the specification. Specifically, the claimed methods have the following general steps: (1) obtaining a sample from a transplant recipient containing dd-cfDNA for both the organ donor and recipient; (2) obtaining genotypes for the organ donor and recipient; (3) multiplex sequencing the sample from the transplant recipients; and (4) using the genotype profiles and the multiplex sequencing results to analyze, detect and

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quantify the dd-cfDNA, and use that information to diagnose, predict, or monitor the transplant status or outcome.

38.   Additionally, the sensitivity of the claimed methods must be greater than 56%, as compared to the sensitivity of the surveillance methods for CAV that were available and used at the time of the invention. As explained in more detail below, a POSA would understand the "current" surveillance method for CAV recited in the claims to be coronary angiography, which was the standard method for monitoring CAV at the time of the invention, and which had a lower sensitivity than 56%.

39.   This interpretation is supported by the intrinsic record. For example, the specification repeatedly explains that the claimed assays have a sensitivity greater than 56%. *See id*. at 2:44-45; 23:31-36. This disclosure in the specification correlates exactly to the claim language requiring a sensitivity greater than 56%, thus demonstrating that the claims simply require that the assays have a sensitivity greater than 56%, which is greater than the sensitivity of the prevailing surveillance method for CAV, coronary angiography. A POSA reading the specification would understand that this sensitivity threshold is one of the novel aspects of the invention that distinguished it from the prior art.

40.   This understanding is confirmed by the prosecution history of the '652 Patent. For example, the claims as originally filed did not recite any sensitivity

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threshold. A0099-102 ('652 Patent file history, May 4, 2012 Claims). The original claims were subsequently cancelled, and the applicant added one dependent claim that required a sensitivity of greater than 56%. A0202-204 at A0204 ('652 Patent file history, March 21, 2013 Claims). While the Examiner rejected the pending claims as obvious, it noted that the prior art did not teach methods that had a sensitivity of greater than 56%. A0205-214 at A0212 ('652 Patent file history, May 10, 2013 Non-Final Rejection).

41.   During a subsequent interview the Applicant and Examiner discussed the Examiner's obviousness rejection "including the broad scope of the independent claim, as well as, proposed limitations in the claimed methods to limit the scope of the claims in order *to capture what the Applicant believes to be the substance and novelty of the invention*." A0103-105 at A0104 ('652 Patent file history, August 23, 2013 Applicant Initiated Interview). Following this Interview, the Applicant amended the independent claims to recite methods wherein the sensitivity is greater than 56%, noting that the cited prior art references did not teach this limitation. A0104-108 at A0107 ('652 Patent file history, October 10, 2013 Applicant Arguments/Remarks Made In Amendment). The Examiner nevertheless rejected the claims as obvious based in part on a new prior art reference, Moreira, which according to the Examiner, taught methods having a sensitivity of greater than 56%.

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A0109-118 at A0113 ('652 Patent file history, November 14, 2013 Final Rejection).[1] The Examiner eventually allowed the claims, after amending them pursuant to a telephonic interview to add the underlined language: "wherein the sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)."   A0119-A0130 at A0125 ('652 Patent file history, February 12, 2014 Notice of Allowance).

42.   The claim language added by the Examiner simply reflects what the Examiner and Applicant understood to be one of the novel aspects of the invention. Specifically, the Examiner and the Applicant understood that then-current methods for surveilling organ rejection, and CAV, had low sensitivities, and that the higher sensitivity of the claimed methods was what made them inventive. As explained in more detail below, a POSA would understand that the claims represent a significant advance over the standard method for surveilling CAV, coronary angiography, which had a sensitivity of only 43-44%.

IX.   Dr. Christians' Opinions Overcomplicate The Clear Claim Language

43.   Even though the claims are clear on their face, Dr. Christians opines that the sensitivity limitation is indefinite because (1) the intrinsic record purportedly

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[1] Dr. Christians also notes the Examiner's acknowledgment that the claimed assays must have a sensitivity greater than 56%. A1093 ¶ 97 ("[I]t appears the Examiner at least understood that a sensitivity greater than 56% would meet the claim limitation.").

does not provide any guidance on how the applicant measured the sensitivity of the claimed assays; (2) the CAV surveillance comparison is nonsensical outside of the context of heart transplants; (3) the intrinsic record does not specify a reference standard for the CAV surveillance methods; and (4) the intrinsic record does not specify the test conditions to determine sensitivity.

44.   In this regard, Dr. Christians appears to suggest that in order for the claims to be definite, the patent must either claim or describe every single testing condition, and a single reference standard to determine the sensitivity of the CAV surveillance assay and the claimed organ transplant rejection assay. I disagree. A POSA would only need a reference standard to determine the sensitivity of the claimed assay, and then compare that to the sensitivity of the standard method for surveilling CAV (i.e., coronary angiography). Additionally, the intrinsic record provides guidance on the testing parameters a POSA could use to determine the sensitivity of the claimed assay, as set forth in more detail below.

A.   A POSA Would Know How To Determine The Sensitivity Of The Claimed Methods

45.   First, Dr. Christians asserts that the written description of the '652 Patent does not provide any information on how the patent applicant measured the claimed diagnostic sensitivity, and that this therefore renders the claims indefinite. I disagree. As Dr. Christians explains in his report, sensitivity is defined as the frequency of predicted positive diagnoses of a condition relative to the number of

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subjects known to have the condition. A POSA at the time of the invention would understand that the reference standard that would be used to confirm the presence of organ rejection and determine the sensitivity of the claimed dd-cfDNA assays would be a biopsy. This is true even today, and is further confirmed by the disclosure in the specification.

46.   In particular, Example 1 in the specification describes a method where dd-cfDNA was used as a marker to detect the onset of organ failure in gender-mismatched heart transplant recipients: "[l]evels of chromosome Y in plasma were monitored at several time points following transplantation for some of these patients, and compared with biopsy time points for organ rejection." D.I. 89-3 at 8:1-21; 25:64-67. Consistent with what a POSA would have understood at the time, in this example, a biopsy was used to confirm whether the patient experienced a rejection episode, with assessment of the level of dd-cfDNA present at the time of a biopsy-determined rejection. *Id.*; *see also* Figs. 3 and 4. A POSA would understand from this example and based on their own knowledge, that a biopsy is the reference standard by which the sensitivity of the claimed assays for detecting organ rejection were and are determined.

47.   This is also consistent with how Natera purported to validate the sensitivity of its own dd-cfDNA testing assay, Prospera®. In particular, Natera evaluated the fraction of dd-cfDNA in 217 *biopsy-matched* plasma samples and

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determined based on these data, that Prospera had a sensitivity of 88.7%, regardless of the type of biopsy or rejection analyzed. *See* A0215-223 at A0220, Altug, Y. et al., *Analytical Validation of a Single-nucleotide Polymorphism-based Donor-derived Cell-free DNA Assay for Detecting Rejection in Kidney Transplant Patients, Transplantation*, 103:12, 2657-65 (December 2019).   This approach is also confirmed by the Bloom paper that Dr. Christians cites elsewhere in his report, and is true regardless of what type of organ transplant a patient has received:

> Although histology obtained via ***needle biopsy remains the standard for diagnosis of rejection***, this technique is infrequently used for surveillance because of the cost, logistics, potential complications, and patient discomfort and inconvenience.
>
> ***
>
> A diagnosis of active rejection was ***confirmed in review of 59 pathologists' biopsy reports***; 58 cases of active rejection in 204 biopsies, performed for clinical suspicion, most commonly an elevation in serum creatinine, and one case of active rejection in 34 surveillance biopsies.

A1126-A1142 at A1127, Bloom, R., et al., *Cell-Free DNA and Active Rejection in Kidney Allografts*, Journal of the American Society of Nephrology, Vol. 28, pp. 2221-2232 (2017) ("Bloom"); *see also*, A1018-1023, Snyder, T., et al., *Universal Noninvasive Detection of Solid Organ Transplant Rejection*, PNAS, vol. 108, no. 15, 6229-34 (April 12, 2011) ("Snyder") at A1018 ("For heart transplant recipients, the gold standard for diagnosis of rejection is the endomyocardial biopsy."); A0224-234, Drachenberg, C.B., et al., *Guidelines for the Diagnosis of Antibody-Mediated*

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Rejection in Pancreas Allografts – Updated Banff Grading Schema, Am. J. Transplantation, 2011; 11: 1792-1802 (May 2011) at A0232 ("Biopsy evaluation remains the gold standard for the diagnosis of [pancreas] allograft rejection."); A0235-242, Wu, T., et al., *A Schema for Histologic Grading of Small Intestine Allograft Acute Rejection*, Transplantation, Vol. 75, 1241-48, No. 8 (Apr. 27, 2003) at A0235 ("The final diagnosis depends on histologic analysis of endoscopy-guided mucosal [intestinal] biopsy specimens."); A0243-248, Demetris, A.J., et al., *Banff Schema for Grading Liver Allograft Rejection: An International Consensus Document*, Hepatology Vol. 25, No. 3 658-63 (1997) at A0244 ("The diagnosis is considered on clinical grounds and confirmed by examination of a core needle biopsy specimen.").

48.   Dr. Christians attempts to distance Example 1 from what a POSA would have known at the time of the invention by asserting that Example 1 applies to digital PCR, while the claimed techniques use multiplex PCR. A1064-1065 ¶ 38. Dr. Christians also asserts that Example 2 contains no discussion of sensitivity whatsoever, and that the specification therefore does not provide any guidance to a POSA on how to calculate sensitivity. I disagree with these opinions. First, as explained above, determining sensitivity is straightforward; it requires comparing an assay to a reference standard to determine the frequency of positive test results for a condition relative to the number of subjects known to have the condition.

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49.   Second, it does not matter that Example 1 describes digital PCR instead of multiplex PCR. What matters is that Example 1 confirms that a POSA would use findings from a biopsy as a reference standard to confirm the presence of organ rejection and determine the sensitivity of the claimed organ transplant surveillance assays.

50.   Additionally, based on the comparison of the biopsy reference standard to levels of dd-cfDNA, Example 1 establishes that "donor derived DNA present in plasma can serve as a potential marker for the onset of organ failure." D.I. 89-3 at 8:18-21; 26:17-19. A POSA would determine the sensitivity of a given assay using biopsy findings as a reference standard, and then perform a simple numerical comparison of the calculated sensitivity of the assay to the sensitivity of the standard method(s) for surveilling CAV.

B.   The Claim Language Makes Sense In The Context Of Any Organ Transplant

51.   Dr. Christians also opines that because the claims are not limited to monitoring rejection of a heart transplant, the required comparison to the sensitivity of methods for detecting CAV in heart transplant patients has no meaning at all outside the context of a heart transplant. A1066 ¶ 41. I disagree with Dr. Christians' opinion, which overcomplicates how a POSA would view the claims. As I explained above, the claims require a simple numerical comparison between the sensitivity of

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the claimed organ transplant surveillance assay and the then-current standard methods for surveillance of CAV (i.e., coronary angiography).

52.   The definition of sensitivity is the same across disease states and across assays, and does not depend on what type of organ transplant one is monitoring. As a simple hypothetical example, one can compare a diabetes drug that is known to be 75% effective, to an arthritis drug that is known to be 99% effective, and conclude that the arthritis drug has a level of effectiveness that is numerically higher than the diabetes drug. This is true regardless of how the two effectiveness percentages were determined, and regardless of the fact that the two drugs are used to treat different disease states. Similarly, one can determine the sensitivity of a heart transplant surveillance method and compare it numerically to the sensitivity of a kidney transplant surveillance method. In sum, all that is required by the claims is a simple numerical comparison of the sensitivity of the claimed assay (regardless of which organ it applies to), with the sensitivity of surveillance methods for CAV that were available and used at the time of the invention. As explained below, a POSA would understand based on the disclosure in the specification that this refers specifically to coronary angiography.

C.   The Intrinsic Record Describes The CAV Comparator Method

53.   I also disagree with Dr. Christians' opinion that the claims are indefinite because they do not identify a reference standard for measuring sensitivity of the

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CAV comparator assays. As an initial matter, Dr. Christians' analysis assumes that the claims cover any method for detecting CAV, and that a POSA would have to determine the sensitivity of that CAV assay by comparing it to a reference standard. According to Dr. Christians, because the written description does not explain which reference standard to use to determine the sensitivity of the CAV assay, the claims are indefinite. I disagree.

54. First, a POSA would understand that the claims do not call for comparing the sensitivity of the claimed dd-cfDNA assays for organ rejection with the sensitivity of any random method for detecting CAV that was known at the time of the invention. Nor do they require determining the sensitivity of any such method for detecting CAV. Instead, as explained in more detail below, a POSA would understand based on the disclosure in the specification and what was known at the time of the invention, that the CAV surveillance methods recited in the claim refer to the standard of care for surveilling CAV, coronary angiography, which had a known sensitivity of 43-44%. In other words, the claims simply reflect that the sensitivity of the claimed assays must have a sensitivity of greater than 56%, which is numerically higher than the 43-44 % sensitivity of coronary angiography for surveilling CAV.

55. As Dr. Christians notes, the claims refer to "current surveillance methods for cardiac allograft vasculopathy (CAV)." As described above, the

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specification discusses two methods for surveilling CAV: coronary angiography and IVUS. A POSA at the time of the invention would understand coronary angiography to be the applicable CAV surveillance method by which to compare the sensitivity of the claimed methods. This is supported by the written description and published literature that would have been available to a POSA.

56.   First, as explained above, the specification discloses that the standard of care, and most commonly applied surveillance method for CAV at the time of the invention, was coronary angiography. D.I. 89-3 at 6:8-11. Dr. Christians does not dispute this in his declaration. A1074 ¶ 60. While the specification also discusses IVUS as a more sensitive alternative method for monitoring CAV, it explains that IVUS was expensive, resource intensive, and associated with significant risk of morbidity and patient discomfort. D.I. 89-3 at 6:12-19.

57.   The fact that coronary angiography was the most commonly applied surveillance method for CAV, and IVUS was a more sensitive but not commonly adopted surveillance method for CAV at the time of the invention, is confirmed by the literature. For example, one publication from 1999 describes coronary angiography as "the routine screening method for cardiac allograft vasculopathy in most transplant centers" but reports that it had inadequate sensitivity compared to IVUS:

> Coronary angiography has remained the most commonly
> used screening method for CAV. Several reports exist,

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> however, on rapid progression from a normal angiogram to severe vasculopathy, that in some cases of sudden death after heart transplantation was diagnosed only at necropsy. IVUS has been demonstrated to be the most sensitive invasive tool for diagnosis of CAV *in vivo*. IVUS, however, does not allow to investigate the complete coronary artery system as it is restricted to major epicardial vessels.

A0249-256 at A0253-254, Spes, C.H., et al., *Functional and Morphological Findings in Heart Transplant Recipients with a Normal Coronary Angiogram: An Analysis by Dobutamine Stress Echocardiography, Intracoronary Doppler and Intravascular Ultrasound*, J. Heart & Lung Transp., 18:5, 391-98 (May 1999).

58.   Additionally, while IVUS was reported to have superior sensitivity compared to coronary angiography, it was more costly, also invasive, and had limited applicability and was therefore not as widely used:

> [D]ue to its lower costs and a definite, albeit small additional risk of IVUS, coronary angiography is more widely used for CAV screening and follow-up during routine transplant surveillance…Because coronary angiography underestimates the prevalence of CAV and the limited availability and applicability of IVUS, additional diagnostic certainty is needed.

A0258-263 at A0259, Stork, S., et al., *Assessment of Cardiac Allograft Vasculopathy Late After Heart Transplantation: When Is Coronary Angiography Necessary?*, J. Heart Lung Transplant, 2006; 25:1103-08, 1104 (September 2006) ("Stork"); *see also* A0264-271, Khan, R. & Jang, I.K., *Evaluation of coronary allograft vasculopathy using multi-detector row computed tomography: a systematic review*,

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Eur. J. of Cardio-Thoracic Surgery, 41 (2012) 415-22 (2012) ("Khan") at A0264

("[I]n most centers, catheter-based coronary angiography (CCA) is performed on an

annual basis...IVUS is the gold standard for detection of CAV, but it is invasive,

limited to large epicardial arteries, and costly, particularly if done on an annual

basis."); A0272-280, Ramzy, D. et al., *Cardiac Allograft Vasculopathy: A Review*,

Can. J. Surg., Vol. 48, No. 4, 319-27 (2005) ("Ramzy") at A0272 ("The diagnosis

of CAV remains a challenge as angiography, the standard method for detecting focal

plaques, lacks sensitivity in detecting CAV, and intravascular ultrasonography, a

more sensitive method, lacks the ability to evaluate the entire coronary tree."). This

is further supported by the 2010 Mehra publication on which Dr. Christians relies,

which contains a published consensus statement on the nomenclature of CAV from

the International Society for Heart and Lung Transplantation. In particular, this

publication notes that "IVUS-detected maximal intimal thickening may be most

useful for its negative predictive value at any time after transplant; however, we do

not see a role for routine IVUS surveillance." The authors further recommend that

the standard nomenclature for the stages of CAV be based by angiographic findings

(i.e., coronary angiograph) coupled with an assessment of heart function (i.e.,

evaluation of ejection fraction). A1169-A1179 at A1170, Mehra MP et al.

International Society for Heart and Lung Transplantation Working Formulation of

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a Standardized Nomenclature for Cardiac Allograft Vasculopathy, J. Heart and Lung Transplantation Vol 29, Issue 7:717-727 (2010) ("Mehra").

59.   Indeed, coronary angiography was still the standard of care as of 2013, while IVUS was still described as a promising alternative with better sensitivity:

> Invasive coronary angiography is the screening and surveillance test of choice for CAV at most cardiac transplant centers and is typically performed on a routine periodic basis. The prevalence of CAV detected by ICA is approximately 10% to 20% at 1 year and 35% to 50% at 5 years. The angiographic diagnosis of CAV has prognostic significance for graft survival, patient survival, and adverse cardiac events. However, concern remains regarding the sensitivity of coronary angiography for CAV when compared with IVUS and histopathologic studies…Although ICA remains the standard of care, studies have raised concern regarding its limited sensitivity for the detection of early-stage CAV.
>
> …
>
> Due to its superior sensitivity, IVUS is now being used by some centers as the diagnostic test of choice for detection of early CAV…Despite its superior sensitivity and the demonstrated prognostic value of intimal thickening, IVUS has not been widely adopted as a routine screening test for CAV due to several limitations. Intimal proliferation assessed by IVUS does not necessarily correlate with small-artery disease by histologic or immunohistochemical analysis. In addition, the size of currently available IVUS catheters makes direct imaging of the distal, small-caliber vasculature technically difficult. Furthermore, multivessel imaging is necessary to optimize the sensitivity of IVUS for detection of CAV.

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A0281-291 at A0284-A0285, Pollack, A, et al., *Detection and Imaging of Cardiac Allograft Vasculopathy*, JACC: Cardiovascular Imaging, (2013) Vol. 6, No. 5; 613-23, 616-17 (May 2013) ("Pollack").

60.   Based on the disclosure in the specification and what was reported in the literature, a POSA would understand that the standard for surveilling CAV at the time of the invention was coronary angiography.   Not only does the literature confirm that coronary angiography lacked the desired level of sensitivity, it reports that coronary angiography had a sensitivity of 43-44%. *See* A0292-A0306, Tuzcu, E.M., *Occult and Frequent Transmission of Atherosclerotic Coronary Disease With Cardiac Transplantation*, Circulation, 91:6, 1706-13 (Mar. 15, 1995) at A0292 ("The *sensitivity* and specificity of coronary angiography were *43%* and 95%, respectively."); A0258-263 (Stork) at A0258 ("Coronary angiography identified CAV correctly *in only 44% of cases*."); A0281-291 (Pollack) at A0284-285 ("Studies comparing ICA with IVUS have demonstrated *sensitivity*, specificity, PPV, and NPV for the diagnosis of CAV that range *from 43% to 44%*, 81% to 95%, 90% to 92%, and 27% to 57%, respectively."). A POSA reading the claims in light of the specification at the time of the invention would therefore understand that coronary angiography had a sensitivity of 43-44%. Using this known sensitivity, the POSA would readily understand that the claims cover assays having a sensitivity of

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56%, which is higher than the 43-44% sensitivity of coronary angiography, the standard CAV surveillance method at the time of the invention.

61.   While the written description and prior art consistently describe coronary angiography as the standard method for detecting CAV at the time of the invention, Dr. Christians nevertheless opines that a number of surveillance methods were developed for the detection of CAV prior to the invention date, and that one would need to determine their sensitivities to make the claimed comparison. To begin, in making this argument, Dr. Christians states that coronary angiography and IVUS were both accepted as the "gold standards" for the detection of CAV at the time of the invention, but that the sensitivities of other methods would be impacted by which of these were chosen as a reference standard. Dr. Christian's analysis in this regard mischaracterizes the claims. The claims do not require using a reference standard for CAV to determine the sensitivity of another CAV assay. All they require is that the claimed assays have a sensitivity greater than 56%, which is higher than the prevailing standard for surveilling CAV at the time of the invention, coronary angiography.

62.   Dr. Christians' assertion that there were many surveillance methods known in the art besides IVUS and CCA that could be used as reference standards is therefore inapplicable. *See* A1080 ¶ 70. Moreover, the publications Dr. Christians cites elsewhere in his report directly contradict this opinion, and confirm

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my analysis above that coronary angiography was the standard for surveilling CAV

at the time of the invention, and that IVUS was an emerging alternative that was not

universally accepted:

> ***Coronary angiography has been the cornerstone of the diagnosis of CAV vasculopathy (CAV)*** before the advent of IVUS. Although coronary angiography is not perfect, it provides a screening tool to grossly detect the presence of CAV.
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> …
>
> [A]lthough ***IVUS remains an experimental tool*** to help investigators evaluate the outcome of various therapeutic conditions, ***clinical utility is limited***, and importantly, may be used at any point in the transplant process for excluding significant disease when the angiogram appears ambiguous. It is unlikely, however, that the IVUS will define flow-limiting epicardial disease that is not demonstrated by a high-quality coronary angiogram. ***Although IVUS remains very sensitive to define CAV, we cannot advocate routine IVUS at this time because its value as a surrogate marker remains investigational***. IVUS holds promise, pending further research, as a guide to therapy as well as a valid surrogate marker.

Mehra at A1170, A1172. For these additional reasons, the claims are not indefinite.

D.   The Claims Are Not Indefinite Based On Test Conditions

63.   Dr. Christians also opines that the sensitivity claim term is indefinite

because the intrinsic record does not provide any objective standards or testing

conditions for (1) the amount of dd-cf-DNA that is indicative of rejection; (2) the

time that has elapsed after transplant; (3) the study design; and (4) the clinical

context. According to Dr. Christians, each of these factors will lead to varying

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sensitivity results depending on which conditions are used, and therefore the claims are indefinite. I disagree with Dr. Christian's opinions.

64.   As an initial matter, Dr. Christians acknowledges that sensitivity has a very straightforward definition that simply requires a comparison of the number of true positives to the total number of subjects with the condition. A1061-1062 ¶ 31. Nevertheless, Dr. Christians opines that sensitivity will vary between settings and patient groups, and cites to STARD guidelines for the reporting of studies of diagnostic accuracy, which he states provide the "essential elements of the design and conduct of a study." A1081 ¶ 74. Dr. Christians appears to suggest that the claims are indefinite because the specification does not disclose each of the items identified in the STARD checklist. I understand that this is not the appropriate inquiry for indefiniteness. In any event, the intrinsic record provides teachings that would guide a POSA's selection of testing conditions to evaluate the sensitivity of the claimed methods, as set forth in more detail below.

1.   The Specification Provides Guidance On The Amount of dd-cfDNA Deemed To Be Indicative Of Organ Rejection

65.   Dr. Christians opines that because the sensitivity of the method will vary depending on the choice of the % dd-cfDNA threshold by which sensitivity is determined, the claims are indefinite. A1083-1084 ¶¶ 76-78. I disagree with Dr. Christians' opinion. To begin, I understand that the fact that results may vary does not render the claims indefinite if a POSA is otherwise able to determine the claim

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scope with reasonable certainty. Nevertheless, the specification provides ample teachings for a POSA to determine the amount of dd-cfDNA deemed to be sufficient to determine whether organ rejection has occurred.

66.   The specification discusses the sensitivity of the claimed methods and notes that "[w]hile the fraction of donor DNA in the recipient blood is currently not well determined and will depend on organ type, one can take 1% as a baseline estimate based on the literature and applicants own studies with heart transplant patients." D.I. 89-3 at 16:62-65. A POSA reading this disclosure would therefore understand that up to 1% would be a reasonable baseline percentage for the amount of dd-cfDNA circulating in a transplant recipient's blood, and that amounts greater than this would signal that organ rejection may be occurring. While the specification teaches that the dd-cfDNA threshold may vary depending on the type of organ one is surveilling, the specification discloses a general baseline 1% dd-cfDNA threshold, and a POSA could determine whether or not to adjust that threshold through standard assessment or routine testing.

67.   Consistent with this teaching, Example 1 in the specification provides a representative embodiment where the percentage of dd-cfDNA in a transplant recipient was monitored over time to determine the status of an organ transplant. *Id.* at 25:40-26:19. Example 1 explains that the "level of chromosome Y detected in plasma was negligible in plasma at three months prior to rejection, but increased >

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10-fold to 2% of total genomic fraction at the time a biopsy determined rejection."

Id. at 26:1-5. Example 1 then cites to Figure 3, which shows that the percentage of dd-cfDNA present in the transplant recipient's plasma started at a baseline percentage of around 1%, and that it increased notably at the time the biopsy detected organ rejection:

Figure 3

Patient 6 Time-Course
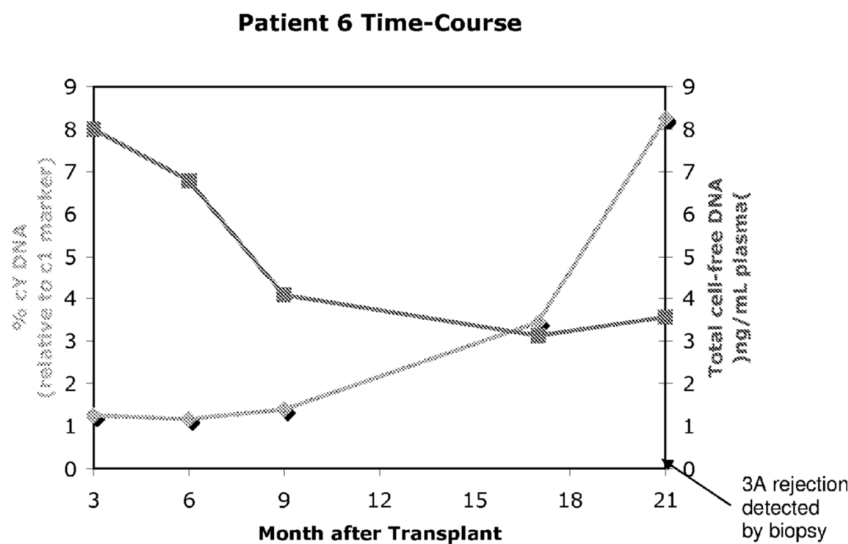
68.   A POSA reading Example 1 would therefore understand that he or she could use a 1% dd-cfDNA as a starting point and baseline threshold to calculate the sensitivity of the claimed assays.

69.   Indeed, the DART Study Dr. Christians cites to show that varying the dd-cfDNA threshold will alter the sensitivity actually further supports that a POSA would choose 1% as the dd-cfDNA threshold to determine sensitivity. Specifically, the authors of the DART Study chose a cutoff of 1.0% dd-cfDNA to evaluate the

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clinical results, including the sensitivity, of the assay used therein. *See* Bloom at A1130. The authors note that "dd-cfDNA may be used to assess allograft rejection and injury; dd-cfDNA levels <1% reflect the absence of active rejection (T cell–mediated type ≥IB or ABMR) and levels >1% indicate a probability of active rejection." *Id*. at A1126; *see also id*. at A1127 ("In stable heart transplant recipients, the fraction of cfDNA originating from the graft is nearly always <1%, whereas during rejection the levels of dd-cfDNA are significantly higher."). The authors concluded based on the data presented in Figure 3 in the Bloom manuscript that Dr. Christians cites that "[w]ith a cutoff of 1.0%, dd-cfDNA had an 85% specificity (95% CI, 79% to 91%) and 59% sensitivity (95% CI, 44% to 74%) to discriminate active rejection from no rejection." *Id*. at A1128. Thus, contrary to Dr. Christians' opinions, the Bloom publication is consistent with what a POSA would readily understand.

70.   Beyond this, even if it were not possible to use 1.0% dd-cfDNA as a starting point and baseline threshold, those of skill in the art would understand that the appropriate cut-off could be determined and optimized empirically and that this was routine statistical application. For instance, a 2006 publication explains as follows:

> When a new diagnostic test is developed or when a diagnostic test is to be used in a clinical condition different from the one for which the test was developed, test's cutoff score may require re-determination. This determination or

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re-determination may usually be based on biological, clinical or demographic situations. Some statistical methods may also be used or may be used in addition to the clinical experiences, analytical and empirical evidences for finding more reliable and valid cutoff point for classifying cases as positive or negative. 95% confidence interval (CI) of mean i.e. Mean ± 2SD method, ROC curve, discriminant function analysis may prove to be helpful statistical tools for such situation.

A0307-310 at A0307, Singh, G., *Determination of Cutoff Score for a Diagnostic Test*, The Internet Journal of Laboratory Medicine, Vol. 2, No. 1 (2006).   One of skill in the art would understand that the diagnostic threshold can be chosen to achieve the desired sensitivities and specificities. *See id.* at A0308 ("Now one may choose a particular observed value of the test as cutoff value, which corresponds to the desired sensitivity and specificity (or 1-specificity).").

71.   Thus, to the extent Dr. Christians contends that the claims are indefinite because the specification does not include a step-by-step method for determining the diagnostic thresholds, I disagree. The person of skill in the art would understand how to do this routinely and would be able to straightforwardly tailor the diagnostic thresholds to particular situations (e.g., different patient groups, different time frames, different organs, etc.) to the extent this is required.

2.   The Specification Provides Guidance To A POSA Regarding When To Measure Sensitivity

72.   I also disagree with Dr. Christians' opinion that the claims are indefinite because sensitivity may vary depending on the amount of time that has elapsed after

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transplantation. A1085-1086 ¶ 80. As I explain above, even if this is true, it would be a routine matter for a POSA to assess the appropriate diagnostic thresholds as a function time after organ transplant and tailor those thresholds to the desired sensitivities and specificities.

73.   Regardless, the written description provides sufficient teachings for a POSA to be able to determine when sensitivity could be measured. First, as explained above, the context of the invention is being able to detect incipient organ rejection to avoid the serious adverse outcomes that occur when organ rejection is detected too late. *See* D.I. 89-3 at 6:19-23 ("Early detection of CAV, prior to the development of angiographically apparent disease, graft dysfunction, or symptom onset is crucial to guide the appropriate use of emerging therapies that retard and occasionally reverse progression of CAV."). Therefore a POSA would be particularly interested in determining the sensitivity of the claimed methods before the onset of symptoms or signs suggesting active rejection.

74.   Consistent with this understanding, the specification explains that the amount of dd-cfDNA circulating in a transplant recipient's blood can be monitored over time and that changes in the % dd-cfDNA can indicate different organ transplant statuses. *See, e.g., id*. at 23:1-12. Again, Example 1 in the specification provides a representative embodiment where dd-cfDNA was measured at various time points following transplantation to determine whether organ rejection was

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occurring.   In particular, Example 1 explains that "[t]he level of chromosome Y detected in plasma was negligible in plasma at three months prior to rejection, but increased >10-fold to 2% of total genomic fraction at the time a biopsy determined rejection. The highest levels of cY in the plasma DNA are seen at this time (FIG.3)." *Id*. at 26:1-6. The specification further explains that similar trends were observed for another patient, the results of which are presented in Figure 4. Figures 3 and 4 in turn show that the % dd-cfDNA had increased substantially when the organ rejection was detected by biopsy 21 and 5 months after transplantation, respectively:
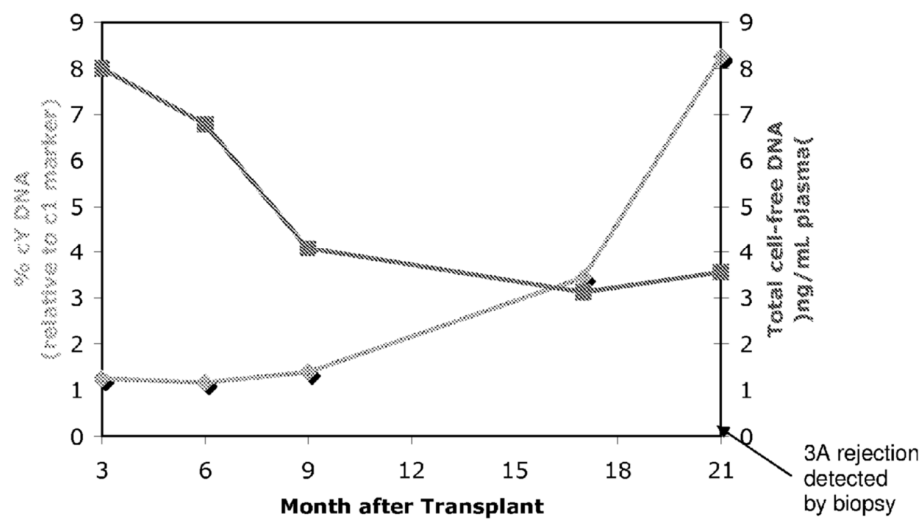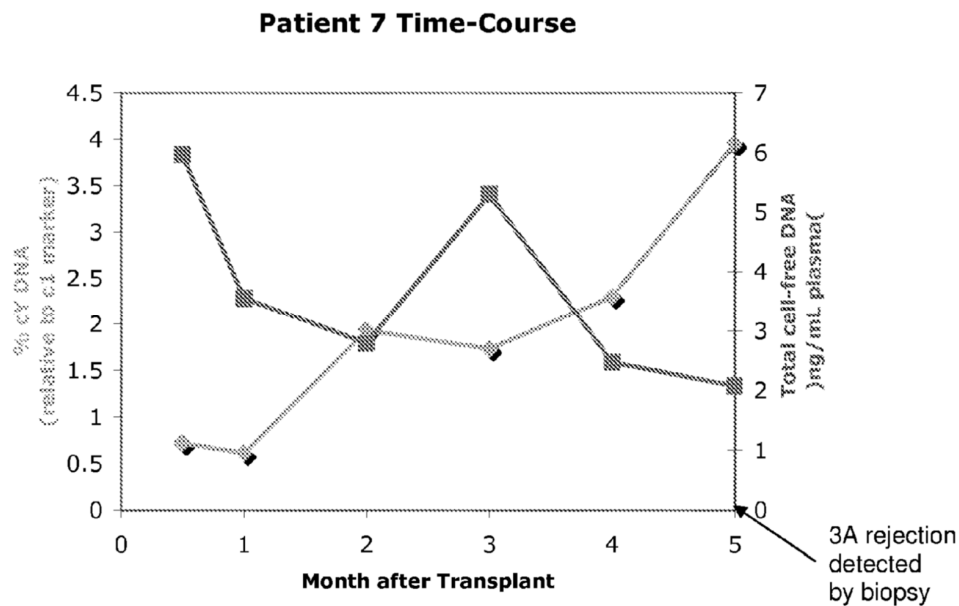
Figure 3

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Figure 4

Patient 7 Time-Course

75. Consistent with the specification's teachings regarding the benefits of detecting organ rejection early on before the development of more serious symptoms, signs, and adverse outcomes, the specification provides specific guidance to a POSA on how to test dd-cfDNA levels following transplantation to properly monitor for organ rejection. For these additional reasons, the claims are not indefinite.

3. Study Design And Clinical Context Does Not Render The Claims Indefinite

76. Finally, I disagree with Dr. Christians' opinion that study design and the clinical context renders the claims indefinite. As an initial matter, many of Dr. Christians' opinions in this section of his report simply rehash his opinions that

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certain variables such as the reference standard, the timing at which dd-cfDNA is measured, and the percentage threshold used will impact sensitivity. *See* A1087-1088, A1091 ¶¶ 84, 85, 91, 92. I disagree with these opinions for the same reasons set forth above.

77.   Dr. Christians also suggests that because the specification does not explain how the claimed sensitivity of greater than 56% was "derived," it cannot validly be compared to the sensitivity of the claimed assays. A1087 ¶ 84. But it does not matter how the sensitivity of 56% was identified as a threshold in the claims and specification. A POSA would readily be able to determine whether the sensitivity of a given assay exceeds 56%, regardless of how the 56% described in the specification was identified.

78.   Dr. Christians' opines that there was "no standard definition for CAV" at the time of the invention, and that different techniques and testing parameters contribute to a lack of standardized methods to diagnose CAV. I disagree with these opinions as well. First, the Mehra article Dr. Christians cites to support these opinions states that CAV is characterized by proliferative thickening of the vascular intima, and provides the clinical consensus on CAV:

> Coronary angiography coupled with assessment of cardiac allograft function maintains the highest level of evidence and consensus opinion for inclusion in the final nomenclature. The advantages of angiography are that it is universal in availability for both adult and pediatric patients, clinically accepted, and applicable at any time in

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the post-transplantation process (favorable for longitudinal and snap-shot assessments).

Mehra at A1170. Therefore, consistent with my explanation above, the Mehra publication confirms that coronary angiography was considered the standard for assessing and, when coupled with an assessment of heart function, defining the stage/progression of CAV, even though different techniques were available. Thus while detection and diagnosis of CAV was not perfect, there was a recognized standard for surveilling CAV and determining CAV status.

79. Additionally, because coronary angiography had a recognized sensitivity of 43-44%, Dr. Christians' criticisms regarding the impact of the severity and type of rejection occurring (A1088 ¶ 86) and the different disease mechanisms that are involved in transplant organ rejection and CAV (A1089 ¶ 87) on the sensitivity of other CAV assays are misguided.

80. As to the type of transplant rejection, the specification discloses that "transplant rejection" encompasses both acute and chronic rejection. D.I. 89-3 at 4:4-35; 21:56-57. According to the specification, acute transplant rejection generally occurs within a few weeks after transplant surgery, while chronic rejection generally occurs within months to years after transplant surgery. *Id*. at 4:12-21. As explained above, Example 1 in the specification provides a representative embodiment that shows a POSA how to measure % dd-cfDNA at various time points after organ transplantation. The specification thus provides the requisite guidance a

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POSA would need to determine the sensitivity of the claimed methods when used to detect acute and/or chronic organ rejection.

81.   In addition to the teachings in the specification, as I explain above, a POSA could determine whether or not to adjust that threshold based on the time at which dd-cfDNA is measured through standard assessment or routine testing. Such techniques could be applied to any category of organ rejection, whether chronic, acute, ABMR, or TCMR.

82.   This guidance is consistent with the teachings in Bloom. For instance, the Bloom paper explains that "[w]ith a cutoff of 1.0%, dd-cfDNA had an 85% specificity (95% CI, 79% to 91%) and 59% sensitivity (95%, CI 44% to 74%) to discriminate active rejection from no rejection." Bloom at A1128. This is confirmed by Figure 3 in the Bloom paper, which Dr. Christians cites in his declaration, and shows that at a dd-cfDNA threshold of 1%, the dd-cfDNA has a sensitivity of over 59% for detecting active rejection (as opposed to sub-categories thereof).

E.   Plaintiffs' Proposed Construction Is Supported And Not Indefinite

83.   Dr. Christians states that Plaintiffs contend that the plain and ordinary meaning of the sensitivity term is "the sensitivity of an infringing method must be at least 56% better than the surveillance methods that were in use at the time of filing for monitoring cardiac allograft vasculopathy ("CAV") disease associated with heart transplant." A1092 ¶ 94. To clarify, I understand Plaintiffs' position is that the

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sensitivity term requires an infringing method to have a sensitivity greater than 56%, which is higher than the reported sensitivity of the standard CAV surveillance method that was in use at the time of the invention. As explained above, the CAV surveillance methods recited in the claims refer specifically to coronary angiography, which had a recognized sensitivity of 43-44%.

84. In this regard, I note that Plaintiffs' proposed construction is otherwise consistent with how Dr. Christians characterizes the claim scope based on the intrinsic record and Dr. Van Ness's testimony. *See id.* ¶ 95 ("By the plain language of the claim, a sensitivity of 60% is 'greater than 56'"); *id.* ¶ 96 ("The written description refers to a claimed method that has at least 56% sensitivity"); A1093 ¶ 97 ("[I]t appears the Examiner at least understood that a sensitivity greater than 56% would meet the claim limitation."); *id.* ¶ 98 ("It is clear that Dr. Van Ness understood that the subject term referred to a sensitivity greater than 56%, not a sensitivity 56% greater than other surveillance methods."); A1094 ¶ 99 ("Dr. Van Ness testified that the claimed methods are directed to an assay with a sensitivity that is better than 56%, not an assay with a sensitivity that is 56% greater than existing methods.").

85. For all of the reasons set forth above, this limitation merely requires that the claimed assay have a sensitivity of greater than 56%, which was higher than the 43-44% sensitivity of the standard for surveilling CAV, coronary angiography.

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X. Conclusion

86. If I am called upon to testify about this report by deposition or at a hearing before the Court, I may use or refer to other documents or information similar to that specifically identified above. While I have not yet prepared any graphics, animations, pictures, demonstrations, or other audio or visual aids, I may use such aids to explain my analysis and opinions.

87. While many of my opinions and analyses are under specific headings, I may use or refer any portion of my report in support for any of my opinions or analysis.

88. I have reviewed documents, files, products, and information in A0093-94 (Appendix B) which I have considered and which form part of the basis for my analysis and opinions in this report. To the extent not identified in A0093-94 (Appendix B), I have also considered each of the documents cited in this report. I am also relying on my expert qualifications and experience for my analysis and opinions.

89. The above opinions and analysis in this report are based upon my consideration of the materials I have reviewed to date. If additional information becomes available, the information and opinions presented may be supplemented or amended.

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Dated: April 1, 2021   By:

Dr. Steven Weisbord

CURRICULUM VITAE

BIOGRAPHICAL INFORMATION

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EDUCATION AND TRAINING
Undergraduate

Dates Attended	Name and Location	Degree	Major Subject
1985-90	University of Rhode Island Kingston, Rhode Island	BA, 1990	Biology
1987	Universidad de Salamanca Salamanca, Spain		Spanish Language and Literature
1987-1988	Tel Aviv University Tel Aviv, Israel		Middle Eastern Studies and Hebrew
1990	University of Granada Granada, Spain		Spanish Language and History

Graduate

Dates Attended	Name and Location	Degree	Major Subject
1993-1997	George Washington University Washington, D.C.	MD, 1997	Medicine
2001-2004	University of Pittsburgh Pittsburgh, PA	MSc, 2004	Clinical Research

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Post-Graduate Residency and Fellowship Training

Dates Attended	Name and Location	Discipline/ProgramDirector
1997-2000	University of Pittsburgh School of Medicine Pittsburgh, PA	Resident Internal Medicine Dr. Frank J. Kroboth
2000-2001	University of Pittsburgh School of Medicine Pittsburgh, PA	Chief Resident Internal Medicine Dr. Frank J. Kroboth
2001-2004	University of Pittsburgh Medical Center Pittsburgh, PA	Fellow, Renal Electrolyte Division Dr. James R. Johnston

APPOINTMENTS AND POSITIONS

2019 -	Professor of Medicine and Clinical and Translational Science (with Tenure) Renal-Electrolyte Division University of Pittsburgh School of Medicine, Pittsburgh, PA
2013-2019	Associate Professor of Medicine (with Tenure) Renal-Electrolyte Division University of Pittsburgh School of Medicine, Pittsburgh, PA
2013-2019	Associate Professor of Clinical and Translational Science Clinical and Translational Science Institute University of Pittsburgh School of Medicine, Pittsburgh, PA
2005-2013	Assistant Professor of Medicine Renal-Electrolyte Division University of Pittsburgh School of Medicine, Pittsburgh, PA
2010-2013	Assistant Professor of Clinical and Translational Science Clinical and Translational Science Institute University of Pittsburgh School of Medicine, Pittsburgh, PA
2004-2005	Visiting Assistant Professor of Medicine Renal-Electrolyte Division University of Pittsburgh School of Medicine, Pittsburgh, PA
2004-Present	Staff Physician Renal Section VA Pittsburgh Healthcare System, Pittsburgh, PA

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2004-Present	Core Faculty Member
	Center for Health Equity Research and Promotion
	Center of Innovation
	VA Pittsburgh Healthcare System, Pittsburgh, PA

2016-Present	Faculty Member
	The Center for Critical Care Nephrology
	Department of Critical Care Medicine
	University of Pittsburgh School of Medicine

CERTIFICATION AND LICENSURE
Specialty certification
Board Certification - Internal Medicine - 2000-2010
Board Certification – Nephrology - through 2024

Other certification
International Society of Nephrology – Clinical Nephropathology Certificate - 2016

Licensure
US Medical License Examination– part 1 June, 1995
US License Examination – part 2 March, 1997
US License Examination – part 3 December, 1997
Medical License - Commonwealth of Pennsylvania
Drug Enforcement Administration: available on request

MEMBERSHIPS in PROFESSIONAL and SCIENTIFIC SOCIETIES
American Society of Nephrology (Fellow 2005)
National Kidney Foundation (Fellow 2011)
International Society of Nephrology

HONORS

University of Rhode Island	Summa Cum Laude, 1990
	Phi Beta Kappa National Honor Society, 1988
	Golden Key National Honor Society, 1989
	Phi Alpha Theta History Honor Society, 1988
	Phi Eta Sigma Freshman Honor Society, 1986
	University College Scholars Award, 1985-1986

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	Children of Alumni Merit Scholarship Award, 1985-1986
	Dean's List, 1985 – 1990
George Washington University	Alpha Omega Alpha Medical Honor Society, 1996
	Kane King Dodek Obstetrics & Gynecology Award, 1997
	National Health Service Corps Scholarship 1993-1997
University of Pittsburgh	Pfizer American Society of Nephrology Fellow Abstract Award, 2002
	Department of Medicine Annual Research Day Post-Doctoral Fellow Research Award, 2003
	Pfizer American Society of Nephrology Fellow Abstract Award, 2003
	Division of Critical Care Medicine Institutional NRSA Awardee 2001-2003
	Conferral of Tenure, 2013
VA Pittsburgh Healthcare System	Bronze medal winner – Pittsburgh Federal Executive Board's 2005 "Rookie of the Year Award in Excellence in Government."
	Raymond M. Rault Faculty Teaching Award 2015-2016

PUBLICATIONS
Refereed Publications

1. **Weisbord SD**, Soule JB, Kimmel PL. Poison on Line - Acute Renal Failure Caused by Oil of Wormwood Purchased Through the Internet. New England Journal of Medicine.1997;337:825-827. PMID: 9297113

2. **Weisbord SD**, Whittle J, Brooks RC. Is warfarin really underused in patients with atrial fibrillation? Journal of General Internal Medicine. 2001;16:743-749. PMID: 11722687

3. **Weisbord SD,** Chaudhuri A, Blauth K, DeRubertis FR. Monoclonal gammopathy and spurious hypophosphatemia. American Journal of the Medical Sciences. 2003;325, 98-100. PMID: 12589234

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4. **Weisbord SD**, Carmody SS, Bruns FJ, Rotondi AJ, Cohen LM, Zeidel ML, Arnold RM. Symptom burden, quality-of-life, advance care planning, and the potential value of palliative care in severely ill hemodialysis patients. Nephrology Dialysis Transplantation. 2003;18:1345-1352. PMID: 12808172

5. **Weisbord SD**, Fried LF, Arnold RM, Rotondi AJ, Levenson DJ, Fine MJ, Switzer GE. Development of a symptom assessment instrument for chronic hemodialysis patients: The Dialysis Symptom Index. Journal of Pain and Symptom Management. 2004; 27(3):226-240. PMID: 15010101

6. **Weisbord SD,** Bruns FJ, Saul MI, Palevsky PM. Provider use of preventative strategies for radiocontrast nephropathy in high-risk patients. Nephron Clinical Practice. 2004; 96(2)C56-62. PMID: 14988599

7. **Weisbord SD**, Fried LF, Arnold RM, Fine MJ, Levenson DJ, Peterson RA, Switzer GE. The prevalence, severity, and importance of physical and emotional symptoms in chronic hemodialysis patients. Journal of the American Society of Nephrology. 2005; 16:2487-2494. PMID: 15975996

8. **Weisbord SD,** Ramkumar M, McBride DJ, Lapidus S, Palevsky PM. Functional Renal Artery Obstruction after Percutaneous Kidney Biopsy. Nephrology Dialysis Transplantation. 2005;20:1274-5. PMID: 15840676

9. Aslam N, Pesacreta M, Bastacky SI, Huston A, Palevsky PM, **Weisbord SD.** Light Chain-Associated Fanconi Syndrome with Nephrotic-Range Proteinuria. American Journal of Kidney Diseases. 2006; 47: e43-45. PMID: 16570336

10. **Weisbord SD,** Chen H, Stone RA, Kip KE, Fine MJ, Saul MI, Palevsky PM. Associations of Increases in Serum Creatinine with Mortality and Length of Hospital Stay Following Coronary Angiography. Journal of the American Society of Nephrology. 2006; 17:2871-2877. PMID: 16928802

11. **Weisbord SD,** Fried LF, Unruh ML, Kimmel PL, Switzer GE, Fine MJ, Arnold RM. Associations of Race with Depression, Symptoms and Spirituality in Maintenance Hemodialysis Patients. Nephrology Dialysis Transplantation. 2007; 22: 2003-2008. PMID: 16998218

12. **Weisbord SD**, Bernardini J, Mor MK, Hartwig KC, Nicoletta PJ, Palevsky PM, Piraino B. The Effect of Intravascular Radiocontrast on Residual Renal Function in Peritoneal Dialysis Patients Undergoing Coronary Angiography. Clinical Cardiology. 2006;29:494-497. PMID: 17133846

13. **Weisbord SD**, Fried LF, Mor MK, Resnick AL, Kimmel PL, Palevsky PM, Fine MJ. Associations of Race and Ethnicity with Hematocrit and Management of Anemia among Patients Initiating Renal Replacement Therapy. Journal of the National Medical Association. 2007;1218-1226. PMID: 18020096

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14. **Weisbord SD**, Fried LF, Mor MK, Resnick AL, Unruh ML, Palevsky PM, Levenson DJ, Cooksey SH, Fine MJ, Kimmel PL, Arnold RM. Renal Provider Recognition of Symptoms in Patients on Maintenance Hemodialysis. Clinical Journal of the American Society of Nephrology. 2007;(5):960-967. PMID: 17702730

15. Carreon M, Fried LF, Palevsky PM, Kimmel PL, Arnold RM, **Weisbord SD**. Clinical Correlates and Treatment of Pain and Sexual Dysfunction in Patients on Maintenance Hemodialysis. Hemodialysis International. 2008;12:268-274. PMID: 18394062

16. **Weisbord SD**, Mor MK, Resnick AL, Hartwig KC, Sonel AF, Fine MJ, Palevsky PM. Prevention, Incidence, and Outcomes of Contrast-Induced Nephropathy. Archives of Internal Medicine. 2008;168:1325-1332. PMID: 18574090

17. **Weisbord SD**, Hartwig KC, Sonel AF, Palevsky PM, Fine MJ. The Incidence of Clinically Significant Contrast-Induced Nephropathy Following Non-Emergent Coronary Angiography - Catheterization and Cardiovascular Interventions. 2008; 71:879-885. PMID: 18412255

18. VA/NIH Acute Renal Failure Trial Network – Intensity of Renal Support in Critically Ill Patients with Acute Kidney Injury. New England Journal of Medicine. 2008;359:82-84 **[Collaborating author].** PMID: 18492867

19. **Weisbord SD**, Mor MK, Resnick AL, Hartwig KC, Palevsky PM, Fine MJ. Incidence and Outcomes of Contrast-Induced AKI Following Computed Tomography. Clinical Journal of the American Society of Nephrology 2008:3:1274-1281. PMID: 18463172

20. **Weisbord SD,** Bossola M, Fried LF, Giungi, S, Tazza L, Palevsky PM, Arnold RM, Luciani G, Kimmel PL. Cultural Comparison of Symptoms in Patients on Maintenance Hemodialysis. Hemodialysis International 2008;12:434-440. PMID: 19090866

21. **Weisbord SD,** Mor MK, Hartwig KC, Palevsky PM, Fine MJ. Factors Associated with the Use of Preventive Care for Contrast-Induced Acute Kidney Injury. Journal of General Internal Medicine. 2009;24:289-298. PMID: 19156472

22. Hanlon JT, Aspinall S, Semla T, **Weisbord SD**, Fried L, Good C, Fine M, Stone R, Pugh MJ, Rossi M, Handler S. Consensus guidelines for oral dosing of primarily renally cleared medications in older adults. Journal of the American Geriatrics Society. 2009;57:335-40. PMID: 19170784

23. Abdel-Kader K, Unruh ML, **Weisbord SD**. Symptom Burden, Depression and Quality of Life in Chronic and End-Stage Renal Disease. Clinical Journal of the American Society of Nephrology. 2009;4:1067-1074. PMID: 19423570

24. Jasuja D, Mor MK, Hartwig KC, Palevsky PM, Fine MJ, **Weisbord SD** – Provider Knowledge of Contrast-Induced Acute Kidney Injury. American Journal of the Medical Sciences 2009;338:280-86. PMID: 19641454

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25. Claxton R, Blackhall L, **Weisbord SD**, Holley JL. Under-Treatment of Symptoms in Patients on Maintenance Hemodialysis. Journal of Pain and Symptom Management. 2010;39:211-218. PMID: 19963337

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28. Nephrotoxicity of Iodixanol versus Iopamidol in patients with chronic kidney disease and diabetes mellitus undergoing coronary angiographic procedures. American Heart Journal. 2009;158:822-828 **[Collaborating author].** PMID: 19853704

29. Hanlon JT, Wang X, Handler SM, **Weisbord SD**, Pugh MJ, Semla, T, Stone RA, Aspinall, SL. Potentially Inappropriate Prescribing of Primarily Renally Cleared Medications in Older Veterans Affairs Nursing Home Patients. Journal of the American Medical Directors Association. 2011:12:377-383. PMID: 21450179

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32. Jhamb M, Pike F, Ramer S, Argyropoulos C, Steel J, Dew MA, **Weisbord SD**, Weissfeld L, Unruh M. Impact of Fatigue on Outcomes in the Hemodialysis (HEMO) Study. American Journal of Nephrology. 2011;33:515-523. PMID: 21555875

33. Green SM, Selzer F, Mulukutla SR, Tadajweski EJ, Green JA, Wilensky RL, Laskey WK, Cohen HA, Rao SV, **Weisbord SD**, Lee JS, Reis SE, Kip KE, Kelsey SF, Williams DO, Marroquin OC. Comparison of Bare Metal and Drug-Eluting Stents in Patients with Chronic Kidney Disease: A Report from the NHLBI Dynamic Registry. American Journal of Cardiology. 2011;108:1658-1654. PMID: 21890077

34. Green JA, Mor MK, Shields AM, Palesky PM, Fine MJ, Arnold RM, **Weisbord SD**. Renal provider perceptions and practice patterns for the management of pain, sexual dysfunction, and depression in hemodialysis patients. Journal of Palliative Medicine. 2012; 15(2):163-7 PMID: 22339326

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28. Yuo T, Wagner J, **Weisbord S**, Dillavou E, Leers S, Hager E. Arteriovenous Fistulae Are Not Always the Lowest Cost Choice for Dialysis Access. 2016; 2.2.1.3. Journal of Vascular Access 17:3

29. Rondon-Berrios H, Tandukar S, Mor MK, **Weisbord SD**. Urea in the Treatment of Hyponatremia: The First Reported US Inpatient Experience. 2018, 71:4:580. American Journal of Kidney Diseases

30. Parikh CR, Palevsky PM, Kaufman JS, Thiessen-Philbrook H, **Weisbord SD**. Kidney Injury Biomarkers and Contrast-Associated AKI: A Sub-Study of the PRESERVE Trial. 2018. American Society of Nephrology – TH-PO1159

PROFESSIONAL ACTIVITIES

Teaching and mentoring

- Biology, chemistry tutoring program – University of Rhode Island – 1991-1992

- Talent Development Program Tutor – University of Rhode Island – 1991-1992

- 1st and 2nd year medical student physical diagnosis course University of Pittsburgh School of Medicine, 2000-2001

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A0074

- Medical student physical diagnosis didactic - University of Pittsburgh School of Medicine, 2000-2001

- Resident clinical report eight month didactic for internal medicine residents - University of Pittsburgh School of Medicine and VA Pittsburgh Healthcare System, 2000-2001

- 2nd year medical student problem-based learning – University of Pittsburgh School of Medicine, 2003

- Medical resident education - renal elective month – University of Pittsburgh School of Medicine, 2003-2004

- Renal grand rounds presentation semi-annually – University of Pittsburgh School of Medicine, 2002-2004

- Renal journal club presentation semi-annually – University of Pittsburgh School of Medicine, 2002-2004

- Renal Physiology block – 2nd year medical student core curriculum – University of Pittsburgh School of Medicine, 2004-Present

- Kidney Course – 3rd year medical student lecture series – University of Pittsburgh School of Medicine, 2004

- Methods and Logic in Medicine – 1st year medical student course – University of Pittsburgh School of Medicine, 2005

- Renal Fellow's weekly lecture series – University of Pittsburgh School of Medicine, 2004-present

- Medical student advisor – Faculty and Students Together (FAST) program – 2005

- Medical student advisor – Fast Fact publication – Prognostication in Dialysis-Dependent CKD, 2007

- Internal Medicine Resident Report – VA Pittsburgh Healthcare System 2004-2006

- University of Pittsburgh School of Medicine 2nd year medical student Renal Block – Small group and pathology session facilitator – 2005-present

- University of Pittsburgh School of Medicine 2nd year medical student lecture – Disorders of volume homeostasis – 2012-present

- Mentor – Dr. Ted Yuo University of Pittsburgh KL2 Awardee – Vascular Access in Hemodialysis Patients 2016-present

- Mentor – Dr. Jamie Green – Nephrology Division Geisinger Medical Center, Danville PA. 2015-2017

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A0075

- Mentor – Dr. Helbert Rondon – University of Pittsburgh School of Medicine – Urea in the Treatment of Hyponatremia due to the Syndrome of Inappropriate Antidiuretic Hormone Secretion. 2017-present

- Chair, PhD Dissertation Committee – Dr. Khaled Abdel-Kader; Clinical and Translational Science, University of Pittsburgh. 2018-present

- Mentor – Dr. Winn Cashion - Evaluation of Healthcare Resource Utilization among Veterans who Receive Solid Organ Transplantation and Related Care within VA, Medicare, or Both Healthcare Systems. 2018 – present

- Mentor – Drs. Hossam Abdalla and Dr. Amogh Puli – Processes of Care for the Management of Erectile Dysfunction in Chronic Hemodialysis Patients – 2017-2018

- Mentor – Dr. Srijan Tandukhar – Does Contrast Media Cause Acute Kidney Injury 2017-present

RESEARCH FUNDING
Current grant support

Grant number	Grant Title	Role in project	Years inclusive	Source $ amount
CSP #578	Prevention of Serious Adverse Events Following Angiography	Principal Investigator 50%	2011-present	VA Cooperative Studies Program $31,061,703
1R01DK106256-01A1	Biomarker Effectiveness Analysis in Contrast Nephropathy (BEACON)	Co-Investigator 3.6%	2016-2021	NIDDK $1,344,963
1RO1DK114085-01	Technology Assisted Stepped Collaborative Care Intervention (TASCCI) to Improve Patient-Centered Outcomes in Hemodialysis Patients	Co-Investigator 5%	2018-2022	NIDDK $2,440,528

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VA VISN 4 CDDF	Management of Acute Coronary Syndrome Among Veterans with CKD	Principal Investigator 12.5%	2019-2021	VA VISN 4 $75,000
1 IO1 HX002738-01	Validation of the GRACE Score to Assess the Appropriateness of Angiography in Veterans with Chronic Kidney Disease	Principal Investigator 12.5%	2019-2020	VA Health Services Research and Development $100,000
U01DK123786	NIDDK Hemodialysis Opioid Prescription Effort (HOPE) Consortium Trial	Co-Investigator 5%	2019-2024	NIDDK $81,506 (local)
COVID19-8900-01	COVID-19 in Veterans with Chronic Kidney Disease	Principal Investigator 12.5%	2020-2021	VA Clinical Science Research and Development $29,411
PAR-18-744	Urea for Chronic Hyponatremia	Co-Principal Investigator 5%	2019-2021	NIDDK $430,375
IO1HX003303	Patterns, Processes and Outcomes of Kidney and Liver Transplantation in the Era of Enhanced Community Care for Veterans	Principal Investigator 37.5%	2021-2024 (approved for funding – April 1, 2021 start date)	VA Health Services Research and Development $1,199,687

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Prior grant support

Grant number	Grant Title	Role in project	Years inclusive	Source $ amount
PEC18-328	Evaluation of Healthcare Resource Utilization among Veterans who Receive Solid Organ Transplantation and Related Care within VA, Medicare, or Both Healthcare Systems	Co-Principal Investigator 3%	2018-2019	VA Quality Enhancement Research Initiative $87,270
1R01DK098214-01	Biomarker Collection and Analysis in the PRESERVE Trial Cohort	Principal Investigator 9%	2013-2018	NIDDK $773,226
R-D2C-1310-07253	A Trial of Sertraline vs. CBT for End-Stage Renal Disease Patients with Depression (ASCEND)	Co-Investigator 5%	2014-2017	PCORI $2,106,602
IIR 07-190	Pain, Sexual Dysfunction, and Depression in Hemodialysis Patients	Principal Investigator 37.5%	2008-2012	VA Health Services Research and Development $1,129,005
5707114	Association of Health Literacy with Clinical Outcomes in Hemodialysis Patients	Co-Investigator 3%	2010-2012	American Kidney Fund $124,792
03-176-2	Pain, Sexual Dysfunction, and Depression in Hemodialysis Patients – Career Development Transition Award	Principal Investigator 75%	2010-2011	VA Health Services Research and Development $428,747
XVA 72-040	Processes of care and outcomes associated with the use of NSAID medications	Principal Investigator 5%	2007-2009	VA VISN 4 $49,896

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A0078

XNV 72-105	Associations of Race with Anemia Management in Veterans with Chronic Kidney Disease	Principal Investigator 3%	2007	Roche $26,135
RCD 03-176-2	Preventing Radiocontrast-Induced Acute Renal Failure	Principal Investigator 75%	2005-2007	VA Health Services Research and Development $339,000
DVX405	A Multicentre, Randomized, Double-Blind, Parallel Group, Phase IV Study to Compare the Renal Effects of the Non-Ionic Iso-Osmolar Contrast Medium Iodixanol 320mgI/mL (Visipaque) with the Non-Ionic Low Osmolar Contrast Medium Iopamidol 370mgI.mL	Site Principal Investigator <5%	2006-2007	GE Healthcare Per patient payment
XNV 72-070	Examining renal provider understanding of chronic hemodialysis patients' symptoms	Principal Investigator 5%	2005-2006	University of Pittsburgh Institute to Enhance Palliative Care $10,000
HFP01-717	Preventing Radiocontrast-Induced Acute Renal Failure	Principal Investigator 5%	2004-2006	VA VISN 4 CPPF $50,000
LIP 72-013	Modification of a Symptom Assessment Scale to Evaluate Symptom Burden in Chronic Hemodialysis Patients	Co-Principal Investigator <5%	2002-2003	VA CHERP $21,000
XVA 72-022	Cardiovascular disease and chronic renal disease in a VA population	Co-Investigator 5%	2001-2003	VA VISN 4 CPPF $49,588

Other research related activities - Invited peer review of manuscripts

- New England Journal of Medicine

- Journal of the American Medical Association

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- American Journal of Medicine

- Archives of Internal Medicine

- Journal of the American College of Cardiology

- Journal of the American Society of Nephrology

- Clinical Journal of the American Society of Nephrology

- Kidney International

- Nephrology Dialysis Transplantation

- American Journal of Kidney Diseases

- American Journal of Nephrology

- Nephron Clinical Practice

- Clinical Nephrology

- Hemodialysis International

- Mayo Clinic Proceedings

- Journal of General Internal Medicine

- Journal of Psychosomatic Research

- Journal of Palliative Care

- Journal of Palliative Medicine

- Journal of Pain and Symptom Management

- Journal of the American Geriatrics Society

- Expert Review of Cardiovascular Therapy

- BMC Blood Disorders

- BMC Nephrology

- Clinical Cardiology

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A0080

- Journal of Intensive Care Medicine

- Advances in Medical Sciences

- Circulation

- Circulation: Cardiovascular Interventions

- Circulation: Cardiovascular Quality and Outcomes

- American Journal of Geriatric Pharmacotherapy

- Future Medicine

- Archives of Medical Research

- Cardiovascular and Interventional Radiology

- Critical Care

- Renal Failure

- Canadian Medical Association Journal

- Medical Principles and Practice

- Nature Reviews Nephrology

- Clinical Transplantation

- International Journal of Impotence Research: The Journal of Sexual Medicine

- Canadian Journal of Behavioral Science

- Computational and Mathematical Methods in Medicine

- American Journal of the Medical Sciences

- International Journal of Cardiology

- American Journal of Cardiology

- Health and Quality of Life Outcomes

- Contemporary Clinical Trials

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A0081

- American Heart Journal

- PLOS Medicine

- Biomed Research International

- Journal of the American Heart Association

- Canadian Journal of Kidney Health and Disease

- Annals of Vascular Surgery

- Journal of Hospital Medicine

Other research related activities - Editorial activities

Editorial Board:

   - Clinical Journal of the American Society of Nephrology (2011-present)

   - Journal of the American Society of Nephrology (2017-present)

Associate Editor – BMC Nephrology (2015-present)

Guest Editor – Advances in Chronic Kidney Disease – Psychosocial and Quality of Life Issues in

    Chronic Kidney Disease – October 2007

Editor – UPMC Renal Grand Rounds – UPMC Renal-Electrolyte Division

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CURRENT RESEARCH INTERESTS

- Risk factors and preventive interventions for contrast-induced acute kidney injury
- Management of patients with chronic kidney disease and acute coronary syndrome
- Quality improvement methodology and its application to acute and chronic kidney disease
- Iatrogenic acute kidney injury
- Symptom burden and quality of life in chronic dialysis patients
- Development and application of clinical practice guidelines
- Management of hyponatremia

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INVITED SEMINARS AND LECTURESHIPS
Local

- Symptom Burden in Hemodialysis Patients – Center for Research on Healthcare – University of Pittsburgh, October 2004

- Contrast-Induced Nephropathy University of Pittsburgh School of Medicine – Department of Medicine Grand Rounds — September 2006

- Update in Nephrology - University of Pittsburgh School of Medicine – Department of Medicine Grand Rounds — October 2006

- Renal Provider Understanding of Chronic Hemodialysis Patients' Symptoms - Center for Research on Healthcare, University of Pittsburgh, May 2007

- Hyperkalemia – VA Pittsburgh Healthcare System Advanced Cardiac Life Support lecture – Pittsburgh, PA, March 2008

- Anemia Management in the Patient with CKD – A Multi-Disciplinary Approach: Chronic Kidney Disease Management for Primary Care Providers, Pittsburgh, PA, September 2008

- Prevention of Contrast-Induced Acute Kidney Injury: The Design of and Justification for a Large, Definitive Trial – University of Pittsburgh Renal-Electrolyte Division research conference, Pittsburgh, PA, November 2009

- Symptoms in Patients on Chronic Hemodialysis – University of Pittsburgh Section of Palliative Care Medicine Grand Rounds, Pittsburgh, Pennsylvania, September 2011

- ATN, Nephron-D, and PRESERVE: An Update on VA Renal Cooperative Studies. University of Pittsburgh Department of Medicine Grand Rounds, Pittsburgh, PA, January 2012

- Prevention of Serious Adverse Events Following Angiography: Primary Trial and Establishment of a Biorepository. 3rd Annual University of Pittsburgh Acute Kidney Injury Symposium – Pittsburgh, PA, October 2013

- Update on the PRESERVE Trial. 5th Annual University of Pittsburgh Acute Kidney Injury Symposium. Pittsburgh, PA, October 2015

- Nephrology Year in Review. Contrast-Associated Acute Kidney Injury: Are We Moving the Field Forward. 2017 University of Pittsburgh School of Medicine/Presbyterian Campus Grand Rounds. Pittsburgh, PA, May 2017

- Nephrology Year in Review. Contrast-Associated Acute Kidney Injury: Are We Moving the Field Forward. 2017 University of Pittsburgh School of Medicine/Shadyside Campus Grand Rounds. Pittsburgh, PA, May 2017

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- Contrast-Associated Acute Kidney Injury: Are We Moving the Field Forward. 2017 Mercy Hospital Grand Rounds. Pittsburgh, PA, May 2017

- Influence of Acute Kidney Injury on Performance of Coronary Angiography in Patients with Chronic Kidney Disease. 8th Annual AKI Symposium. Department of Critical Care Medicine, University of Pittsburgh School of Medicine. Pittsburgh, PA, October 2018

- Contrast-Induced Nephropathy. East by Southwest Renal Symposium. Pittsburgh, PA, September 2019

- Source of Post-Kidney Transplant Care and Long-Term Mortality – Starzl Transplant Institute Grant Rounds – January 2021

Regional
- Contrast Induced Nephropathy: Clinical Importance and Prevention - Pinnacle Health System Department of Medicine Grand Rounds — Harrisburg, PA - April 2007

- Prevention of Contrast-Induced Nephropathy with Volume Expansion - Contrast-Induced Nephropathy roundtable – New York, New York – September 2006

- Global Symptom Assessment: Why and How – Mid-Atlantic Renal Coalition End of Life – Pain Management Meeting – Washington, D.C., June 2007

- Epidemiology and Outcomes of Contrast-Induced Nephropathy – Grand Rounds – The Reading Hospital, Reading, PA, April 2008

- ANCA-Associated Renal Disease – Chief of Medicine conference – VA Pittsburgh Healthcare System, Pittsburgh, PA, June 2008

- Contrast-Induced Nephropathy – Cardiology Grand Rounds – Mount Clemens General Hospital, Detroit, MI, August 2008

- Academic Nephrology: Lessons Learned by a Junior Investigator – Mid-Atlantic Young Nephrology Investigators Forum, Baltimore MD, March 2009

- Update on the Impact of Iodinated Contrast Media in Interventional Procedures – Continuing Medical Education Presentation – Advocate South Suburban Hospital, Chicago, IL, June 2009

- Sodium Bicarbonate for the Prevention of Acute Kidney Injury Following Cardiac Surgery – Lessons Learned and Future Opportunities – Tribe AKI meeting – Toronto, Canada, October 2009

- Contrast-Induced Nephropathy, Nephrogenic Systemic Fibrosis, and Acute Phosphate Nephropathy – Annual meeting of the New Mexico Chapter of the American College of Physicians – Albuquerque, NM, October 2009

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A0084

- Assessment and Management of Symptom in Patients on Dialysis – Kidney End-of-Life Coalition/Mid-Atlantic Renal Coalition Webinar, March 2010

- Assessment and Management of Symptoms in ESRD – 2010 Meeting of The Renal Network, Hershey, Pennsylvania, April 2010

- Case discussant – Chronic Renal Insufficiency is the New Diabetes – From Controversy to Consensus in Cardiovascular Care, Farmington, Pennsylvania, June 2010

- Case discussant – Screening, Diagnosis and Therapy in Renal Artery Stenosis - From Controversy to Consensus in Cardiovascular Care, Farmington, Pennsylvania, June 2010

- Case discussant — Diabetes and Chronic Renal Disease: Strategies in Managing Coronary Artery Disease and Risk - From Controversy to Consensus in Cardiovascular Care, Farmington, Pennsylvania, May 2011

- Symptoms in Dialysis Patients. University of New Mexico School of Medicine, Nephrology Division Grand Rounds. Albuquerque, NM, February 2015

- Significance, Clinical Implications, and Prevention of Contrast-Induced Acute Kidney Injury. University of New Mexico School of Medicine, Department of Medicine Grand Rounds. Albuquerque, NM, February 2015

- Contrast Nephropathy: Scanning the Evidence. University of Cincinnati Acute Care Nephrology Symposium. Cincinnati, OH, May 2017

- The PRESERVE Study: Trials, Tribulations and Implications. 2018 Yale University TRenal Symposium. New Haven, CT. May 2018

National
- Use of warfarin in patients with atrial fibrillation – 2000 Annual Meeting of the Society of General Internal Medicine. Boston, Massachusetts

- Are intravenous fluids used to limit the risk of contrast nephropathy? – 2002 Annual Meeting of the American Society of Nephrology – Pfizer Fellow Award Ceremony. Philadelphia, Pennsylvania

- Defining clinically significant radiocontrast nephropathy – 2003 Annual Meeting of the American Society of Nephrology – Pfizer Fellow Award Ceremony. San Diego, California

- Quality of Life and Symptoms in Elderly ESRD Patients – 2007 Annual Meeting of the American Society of Nephrology, San Francisco, Ca, November 2007

- Symptoms in Patients with End-Stage Renal Disease – 2008 Annual Meeting of the National Kidney Foundation – Dallas, Tx, April 2008

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A0085

- How Common is Clinically Significant Contrast-Induced Nephropathy Following Computed Tomography? – Stanford University's 10th Annual International Symposium on Multi-Detector-Row CT, Las Vegas, NV, May 2008

- The Role of Intravenous Fluids for the Prevention of Contrast-Induced Nephropathy - Stanford University's 10th Annual International Symposium on Multi-Detector-Row CT, Las Vegas, NV, May 2008

- Is Oral Hydration Effective for the Prevention of Contrast-Induced Nephropathy? - Stanford University's 10th Annual International Symposium on Multi-Detector-Row CT, Las Vegas, NV, May 2008

- Contrast-Induced Acute Kidney Injury – 2008 Annual Meeting of the American Society of Nephrology, Critical Care Nephrology Course, Philadelphia, PA, November 2008

- Contrast-Induced Acute Kidney Injury: Mortality Risk and Prevention – 2009 Annual Meeting of the National Kidney Foundation, Nashville TN, March 2009

- Pain in ESRD - 2009 Annual Meeting of the National Kidney Foundation, Nashville TN, March 2009

- Management of the Critically Ill ESRD Patient - 2009 Annual Meeting of the National Kidney Foundation, Nashville TN, March 2009

- Assessment and Management of Symptoms in Patients with End-Stage Renal Disease – National Hospice and Palliative Care Organization annual meeting – Denver, CO, September 2009

- Contrast-Induced Acute Kidney Injury - 2009 Annual Meeting of the American Society of Nephrology, Critical Care Nephrology Course, San Diego, CA, October 2009

- Quality of Life in ESRD Patients: The Burden and Role of Physical Symptoms – 2010 Annual Meeting of the National Kidney Foundation, Orlando, Florida, April 2010

- Which Patients Are at Risk for Clinically Significant Contrast-Induced Nephropathy Following Computed Tomography – 2010 International Symposium on Multidetector Row Computed Tomography, San Francisco, California, May 2010

- Assessing Renal Function Prior to Computed Tomography to Determine Risk for Contrast-Induced Nephropathy - 2010 International Symposium on Multidetector Row Computed Tomography, San Francisco, California, May 2010

- Contrast Induced Acute Kidney Injury – Can We Prevent It and Does it Matter? 2010 Annual Meeting of the American Society of Nephrology, Denver, Colorado, November 2010

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A0086

- Bicarbonate, Saline, and NAC in the Prevention of Contrast-Induced Acute Kidney Injury: An Analysis of the Meta-Analyses - 2010 Annual Meeting of the American Society of Nephrology, Denver, Colorado, November 2010

- Symptoms in Non-Dialysis Dependent CKD - 2010 Annual Meeting of the American Society of Nephrology, Denver, Colorado, November 2010

- Prevention of Contrast-Induced Acute Kidney Injury – 2011 Annual Meeting of the National Kidney Foundation, Las Vegas, Nevada, April 2011

- Contrast Induced Acute Kidney Injury – Can We Prevent It and Does It Matter? 2011 Annual Meeting of the American Society of Nephrology, Philadelphia, PA, November 2011

- Contrast Induced Acute Kidney Injury – Can We Prevent It and Does It Matter? 2011 Annual Meeting of the American Society of Nephrology, San Diego, CA, October 2012

- Contrast Induced Acute Kidney Injury – Can We Prevent It and Does It Matter? 2013 Annual Meeting of the American Society of Nephrology. Atlanta, GA, November 2013

- Using Biomarkers in the Management and Prognosis of Contrast-Induced Acute Kidney Injury. Arnold O. Beckman Conference – American Association for Clinical Chemistry and American Society of Nephrology. Atlanta, GA, 2013

- Pain in Hemodialysis Patients. National Kidney Foundation Spring Clinical Meeting. Las Vegas, NV, 2014

- Thinking Straight – Cognitive Function in Diabetes and Chronic Kidney Disease – 2014 Annual Meeting of the American Diabetes Association. San Francisco, CA 2014

- The Critically Ill End-Stage Renal Disease Patient – 2014 Annual Meeting of the American Society of Nephrology. Philadelphia, PA, November 2014

- Contrast-Induced Acute Kidney Injury: Can We Prevent It? Does It Matter? 2014 Annual Meeting of the American Society of Nephrology. Philadelphia, PA, November 2014

- Interventions to Alleviate the Symptom Burden in Dialysis Patients. 2014 Annual Meeting of the American Society of Nephrology. Philadelphia, PA, November 2014

- The Critically Ill End-Stage Renal Disease Patient – 2015 Annual Meeting of the American Society of Nephrology. San Diego, CA, November 2015

- Contrast-Induced Acute Kidney Injury: Can We Prevent It? Does It Matter? 2015 Annual Meeting of the American Society of Nephrology. San Diego, CA, November 2015

- Endpoints in AKI Trials. 2015 Annual Meeting of the American Society of Nephrology. San Diego, CA, November 2015

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A0087

- The Critically Ill End-Stage Renal Disease Patient – 2016 Annual Meeting of the American Society of Nephrology. Chicago, Ill, November 2016

- Contrast-Induced Acute Kidney Injury: Can We Prevent It? Does It Matter? 2016 Annual Meeting of the American Society of Nephrology. Chicago, Ill, November 2016

- The Critically Ill End-Stage Renal Disease Patient – 2017 Annual Meeting of the American Society of Nephrology. New Orleans, November 2017

- Contrast-Induced Acute Kidney Injury: Can We Prevent It? Does It Matter? 2017 Annual Meeting of the American Society of Nephrology. New Orleans, November 2017

- The PRESERVE Trial. 2017 American Heart Association Scientific Sessions. Anaheim, CA. November 2017

- The Critically Ill End-Stage Renal Disease Patient – 2018 Annual Meeting of the American Society of Nephrology. San Diego, CA October 2018

- Contrast-Induced Acute Kidney Injury. 2018 Annual Meeting of the American Society of Nephrology. San Diego, CA. October 2018

- The Critically Ill End-Stage Renal Disease Patient – 2019 Annual Meeting of the American Society of Nephrology. Washington DC. November 2019

- Interventions to Prevent Contrast-Associated Acute Kidney Injury – 2019 Annual Meeting of the American Society of Nephrology. Washington DC. November 2019

- The Critically Ill End-Stage Renal Disease Patient – 2020 Annual Meeting of the American Society of Nephrology.

- Contrast Associated Acute Kidney Injury – Does it Exist and How Can We Prevent It? – 2020 Annual Meeting of the American Society of Nephrology.

- Disparities in utilization of diagnostic and therapeutic angiography in patients with CKD. NKF/ASCAI Scientific Symposium – Management of Patients with Chronic Kidney Disease in Need of Cardiovascular Catheterization – September 2020

International
- Making Sense of the Evidence with Contrast-Induced Nephropathy - World Congress of Nephrology Symposium — Rio de Janeiro, Brazil, April 2007

- Contrast Induced Nephropathy from a Nephrologist's Perspective – European Renal Association Symposium – Barcelona, Spain, June 2007

- Contrast-Induced Nephropathy – Outcomes and Clinical Consequences – Annual Meeting of the Mexican Society of Radiology and Imaging, Mexico City, Mexico - January 2008

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A0088

- Contrast-Induced Nephropathy – Outcomes and Prevention – Annual Meeting of the Latin American Congress of Endovascular Therapy, Guadalajara, Mexico – February 2008

- Nephrogenic Systemic Fibrosis and the Role of Gadolinium-Based Contrast Agents in Magnetic Resonance Practice: Patient Based Management Strategies Annual Meeting of the European Society for Magnetic Resonance in Medicine and Biology, Valencia, Spain, October 2008

- Understanding Risk Factors for NSF to Guide Preventive Care in Patients with Kidney Disease – 2009 Annual meeting of the European Society of Radiology (ECR), Vienna, Austria March 2009

- NSF: Understanding Causal Mechanisms and Patient Based Management Strategies – World Congress of Nephrology, Milan, Italy May 2009

- CT or MRI in the Patient with Advanced CKD: Is There a Right Answer? – World Congress of Nephrology, Milan, Italy, May 2009

- Trials and Tribulations in AKI: Do We Know Where We Are Going? At the Limits: Cardiology, Diabetes and Nephrology. Cape Town, South Africa, April 2017

- Trials and Tribulations in AKI: Do We Know Where We Are Going? 2019 At the Limits: Cardiology, Diabetes and Nephrology. Campinas, Brazil, July 2019

SERVICE

University of Pittsburgh School of Medicine
- Key Event Committee Member – Bypassing the Blues – A Study to Improve the Quality of Life Following Cardiac Bypass Surgery – June 2007

- Western Pennsylvania Institute and Clinic Research Committee member
- Medical Student Interviewer, University of Pittsburgh School of Medicine

- Chair – Data Safety Monitoring Committee – Protocolized Diuretic Strategy in Cardiorenal Failure (ProDiuS) Trial – University of Pittsburgh School of Medicine

- Internal Scientific Grant Reviewer - University of Pittsburgh Center for Research on Healthcare 2012-2013

- Renal faculty organizer - Renal-Electrolyte Division Pathology conference 2015-present

- Member – University of Pittsburgh University School of Medicine Renal-Electrolyte Division Fellow Competency Committee 2016-present

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A0089

- Member – University of Pittsburgh Department of Medicine Promotions and Tenure Committee 2016-present

- Member – University of Pittsburgh School of MedicineTenured Faculty Promotions and Appointments Committee 2018-2020

- Grant Reviewer – University of Pittsburgh Competitive Medical Research Fund 2019

VA Pittsburgh Healthcare System

- Core Member – Institutional Review Board, VA Pittsburgh Healthcare System 2006-2009

- Alternate voting member – Research Scientific Evaluation Committee (RSEC) – VA Pittsburgh Healthcare System. 2010-2013

- VA Pittsburgh Transfusion Committee 2016-present

- Ad hoc member – VA Pittsburgh Healthcare System Medical Executive Board 2017-2018

- Member – VA Pittsburgh Healthcare System Clinical Systems Improvement Committee 2019

Other service

- Grant Reviewer - National Institutes of Health — Renal and Urological Sciences Integrated Review Group. 2008

- Abstract reviewer – 2006 Annual meeting of the American Society of Nephrology

- Expert Panel Member – Mid-Atlantic Renal Coalition End of Life and Pain Management Meeting – Developing an algorithm for the treatment of pain in ESRD - Washington, D.C. 2007

- Expert Interview – Association of Race with Use of Erythropoiesis-Stimulating Agents: An Expert Interview with Steven D. Weisbord MD – Medscape Nephrology – San Francisco, CA. 2007

- Faculty Member and Reviewer – Faculty of Medicine 1000. 2008

- Faculty judge – Mid-Atlantic Young Nephrology Investigators Forum – Baltimore, MD. 2009

- Chair – Workgroup to revise the Renal Physicians Association and American Society of Nephrology Clinical Practice Guideline on Shared Decision-Making in Appropriate Initiation of and Withdrawal from Dialysis – Estimating Prognosis in Acute Kidney Injury

- Consultant – Prognosis and Dialysis Project/Grant – Baystate Health, Springfield, MA

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A0090

- Department of Veterans Affairs – Member - Merit Review Sub-Committee for Clinical Trials. 2009

- Chair – Abstract group – Dialysis: Dialysis for Acute Kidney Injury: hemodialysis, CRRT, SLED, others – 2010 Annual Meeting of the American Society of Nephrology

- Invited small group moderator – Acute Kidney Injury Clinical Trial Design Workshop – National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD. 2010

- Abstract reviewer – 2011 Annual meeting of the American Society of Nephrology

- Invited committee member – National Kidney Foundation KDOQI commentary on KDIGO Acute Kidney Injury guidelines

- Patient Centered Outcomes Research Institute Pilot Project/NIH/CSR – Grant Reviewer. 2012

- Abstract reviewer – 2012 Annual meeting of the American Society of Nephrology

- Moderator – American Society of Nephrology 2012 Annual meeting – "On the Margins: Palliative Care, Treatment Decision Making, and Patient Safety" – San Diego, CA. 2012

- Member – Data Safety Monitoring Committee - Time to Reduce Mortality in End-Stage Renal Disease (TiME) Trial. 2015-2017

- Abstract reviewer – 2013 Annual meeting of the American Society of Nephrology

- Member – KDIGO Supportive Care Conference Symptom Work Group. 2013

- Moderator – American Society of Nephrology 2013 Annual Meeting. "The Good, the Bad, and the Ugly. Evaluating Clinical Trials in Nephrology - Atlanta, GA, 2013

- Grant Reviewer - NIH ZDK1GRB-J J21, RFA DK13-008 USRDS Special Study Centers (U01)

- Member – Data Safety Monitoring Committee – Shared Decision Making and Renal Supportive Care Trial

- Invited participant – Rogosin Institute symposium – Mental Health Issues in Patients with Kidney Disease – New York, New York. 2015

- Member – VA Pittsburgh Healthcare System Transfusion Committee 2016-present

- Grant Reviewer - National Institutes of Health – Special Emphasis Panel/Scientific Review Group: Small Business: Instrumentation, Environmental and Occupational Health. 2016

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A0091

- Invited reviewer – Contrast-Associated Acute Kidney Injury – American Society of Nephrology and John M. Eisenberg Center for Clinical Decisions and Communications Science Center for Collaborative and Interactive Technologies (CCIT) Baylor College of Medicine. 2017

- Member – Kidney Health Initiative - Prioritizing Symptoms of ESRD Patients for Developing Therapeutic Interventions Workgroup. 2016-2017

- Abstract reviewer – 2016 Annual meeting of the American Society of Nephrology

- Member – National Kidney Foundation Care Partner Work Group. 2018

- Member – VA Advisory Committee – Reducing Kidney Disease Through AKI Prevention. 2018-2020

- Abstract Judge – University of Pittsburgh Department of Medicine Research Day 2019

- Member – Technical Expert Panel – Veterans Health Administration Evidence Review on Screening and Treatment of Depression in Veterans with End-Stage Kidney Disease – 2019

- Co-Chair – Management of Patients with Kidney Disease in Need of Cardiovascular Catheterization Symposium - 2020

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A0092

List of Materials Considered
Articles, Papers, and Publicly Available Information:
Altug, Y., et al. "Analytical Validation of a Single-nucleotide Polymorphism-based Donor-derived Cell-free DNA Assay for Detecting Rejection in Kidney Transplant Patients" Transplantation Journal, Vol. 103, Number 12, February 1, 2019
Bloom, R., et al. "Cell-Free DNA and Active Rejection in Kidney Allografts," Journal of the American Society of Nephrology, Vol. 28, pp. 221-2232 (2017)
Demetris, A., et al. "Banff Schema for Grading Liver Allograft Rejection: An International Consensus Document", Hepatology, February 9, 1996
Drachenberg, C., et al. "Guidelines for the Diagnosis of Antibody-Mediated Rejection in Pancreas Allografts - Updated Banff Grading Schema", American Journal of Transplantation, May 17, 2011
Fitzgerald, P, et al., "Intravascular Ultrasound Imaging of Coronary Arteries Is Three Layers the Norm?", Brief Rapid Communication, Intracorrnary Ultrasound Imaging, Vol. 86, No, 1 , March 27, 1992
Khan, R., Jang, I, "Evaluation of coronary allograft vasculopathy using multi-detector row computed tomography: a systematic review", European Journal of Cardio-Thoracic Surgery 41 (2012) 415–422
Kobashigawa, J., et al., "Multicenter Intravascular Ultrasound Validation Study Among Heart Transplant Recipients", Journal of the American College of Cardiology, Vol. 45, No. 9, February 14, 2005
Mehra, M., et al., "International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy - 2010," The Journal of Heart and Lung Transplantation, Vol. 29, No. 7, pp. 717-727 (2010)
Pollack, A., et al. "Detection and Imaging of Cardiac Allograft Vasculopathy" Imaging of Cardiac Allograft Vasculopathy, Vol. 6, No. 5, May 2013, 613-23
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A0093

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Patents and File Histories:
U.S. Patent No. 8,703,652
U.S. Patent No. 8,703,652 FH
Deposition Transcripts and Exhibits:
2021-03-29 Deposition Transcript of Uwe Christians
Other:
Declaration of John F. Beausang in Support of Application No. 13/508,318
2021-03-09 Declaration of Uwe Christians in Support of Defendant Eurofins Viracor Inc.'s Answering Claim Construction Brief and materials cited therein

A0094

PATENT

Attorney Docket No. STAN-706

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application:	Confirmation No.: 6003
Inventor: Stephen R. Quake, et al.	Examiner: Amy M. Bunker
Application No.: 13/508,318	Group Art Unit: 1639
Filed: July 19, 2012	Customer No.: 77974
Title: **NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS**	

FILED ON: January 30, 2014

DECLARATION UNDER 37 C.F.R. § 1.132

Dear Madam:

I, JOHN F. BEAUSANG, declare as follows:

1.   I am currently a Scientist II at ImmuMetrix, Inc.

2.   From 2011-2013, I was a post-doctoral fellow in the laboratory of Stephen R. Quake at Stanford University, where I studied high throughput sequencing applications in genomics, cancer biology and immunology.

3.   I am familiar with the prosecution history of the above-identified patent application.

4.   I am submitting this declaration, and the attached Exhibit A, to demonstrate that genotype information obtained from a transplant recipient can be used to establish a polymorphism profile to detect donor-derived cell-free nucleic acids in a sample from the transplant recipient.

5.   The attached Exhibit A is a figure showing an analysis that I performed showing the detection of donor-derived cell-free DNA based on genotype information obtained from a transplant recipient. The figure reflects an analysis that I performed on sequencing and genotyping data generated by others.

6.   The underlying data for the figure was obtained from banked plasma samples from transplant recipients at Stanford University hospital. DNA from the samples was purified using commercially available kits. Sequencing libraries were constructed from cell-free DNA using

Page 1 of 4

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Application No.: 13/508,318
Declaration Under 37 C.F.R. § 1.132

commercially available kits and sequenced following standard protocols using an Illumina GAII sequencer. Genotypes of the transplant recipients were obtained from genomic DNA using the Illumina Omni1-Quad Beadchip following standard protocols. Data was analyzed using a combination of commercial, publicly available and custom computer code. For additional details see T.M. Snyder et al., 2011. Universal noninvasive detection of solid organ transplant rejection. *Proceedings of the National Academy of Sciences USA*, 108(15): 6229-6234.

7. The attached Exhibit A shows that the time of peak donor-derived, cell-free DNA in the transplant recipient was 5 months. The time points shown in Exhibit A correspond to time points of when biopsies were performed on the transplant recipient. At 5 months, rejection was detected by biopsy and the transplant recipient was treated for transplant rejection (for additional details see T.M. Snyder et al., 2011. Universal noninvasive detection of solid organ transplant rejection. *Proceedings of the National Academy of Sciences USA*, 108(15): 6229-6234). Thus, Exhibit A shows that detection of donor nucleic acids based on genotype information from a transplant recipient can be used to diagnose, predict or monitor a transplant status or outcome.
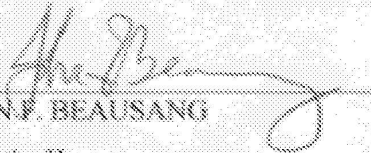
8. Based on the results described in Example 1 of the Specification and the results from the attached Exhibit A, I believe the method of claim 36 would work with genotype information from the donor, genotype information from the transplant recipient or genotype information from both the donor and the transplant recipient. Information from the genotype data can be used to determine which SNPs in the donor differ from the recipient by at least one allele. These sites can then be used to identify and tally donor-specific and recipient-specific sequencing reads from the cell-free DNA, and to observe the increase in donor DNA during rejection events. In the event that data for only one genotype is available (either the donor or the recipient) the genotype of the other can be inferred from publicly available databases (e.g., dbSNP) that report the most likely allele frequencies in the population at each SNP site. For example, if the recipient genotype at a particular SNP site is known to be AA, the database reports 50%A and 50%C, and the sequencing data from cell-free DNA reports a 'C' then, even if the donor genotype was not measured, it can be in inferred with high likelihood that this sequencing read can be attributed to the donor since it matches the database.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

ImmuMetrix 39820-702 831 Inventor Declaration under Rule 132_(pahb)_6099309_1)_clean3FB.docx   Page 2 of 4

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Application No.: 13/508,318
Declaration Under 37 C.F.R. § 1.132

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001.

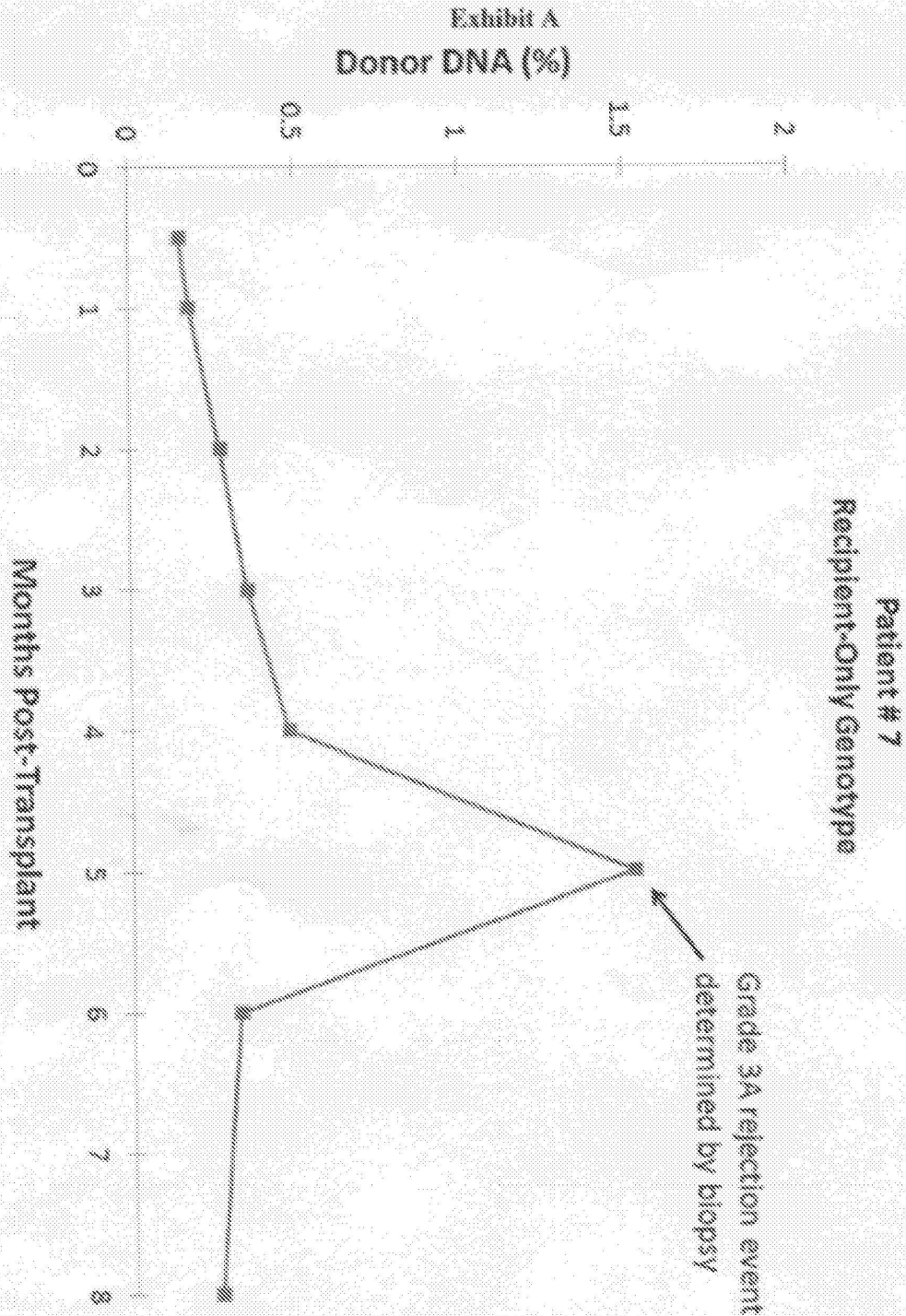Executed on this 30th day of January 2014.

JOHN F. BEAUSANG

Scientist II

ImmuMetrix, Inc.

3183 Porter Drive

Palo Alto, CA 94025

A0097

Application No.: 13/568,318
Declaration Under 37 C.F.R. § 1.132

Exhibit A

Donor DNA (%)

Patient # 7
Recipient-Only Genotype

Grade 3A rejection event
determined by biopsy

Months Post-Transplant

Page 4 of 4

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WHAT IS CLAIMED IS:

1. A method of diagnosing or predicting transplant status or outcome comprising:

providing a sample from a subject who has received a transplant from a donor;

determining the presence or absence of one or more nucleic acids from said donor transplant, wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile; and

diagnosing or predicting transplant status or outcome based on the presence or absence of said one or more nucleic acids.

2. The method of claim 1 wherein said transplant status or outcome comprises rejection, tolerance, non-rejection based allograft injury, transplant function, transplant survival, chronic transplant s injury, or titer pharmacological immunosuppression.

3. The method of claim 2 wherein said non-rejection based allograft injury is selected from the group of ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

4. The method of claim 1 wherein said sample is selected from the group consisting of blood, serum, urine, and stool.

5. The method of claim 1 wherein said marker profile is a polymorphic marker profile.

6. The method of claim 1 wherein said polymorphic marker profile comprises one or more single nucleotide polymorphisms (SNP's), one or more restriction fragment length polymorphisms (RFLP's), one or more short tandem repeats (STRs), one or more variable number of tandem repeats (VNTR's), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, or one or more insertion elements.

7. The method of claim 1 wherein said polymorphic marker profile comprises one or more SNPs

8. The method of claim 1 wherein said transplant is selected from the group consisting of kidney transplant, heart transplant, liver transplant, pancreas transplant, lung transplant, intestine transplant and skin transplant.

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A0099

9. The method of claim 1 wherein said nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA and RNA hairpins.

10. The method of claim 1 wherein said nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA and cDNA.

11. The method of claim 1 wherein said nucleic acid is mRNA.

12. The method of any of the claims 9 to 11 wherein said nucleic acid is obtained from circulating donor cells.

13. The method of claim 1, wherein said nucleic acid is circulating cell-free DNA.

14. The method of claim 1, wherein the presence or absence of said one or more nucleic acids is determined by a method selected from the group consisting of sequencing, nucleic acid array and PCR.

15. The method of claim 14 wherein said sequencing is shotgun sequencing.

16. The method of claim 14 wherein said array is a DNA array.

17. The method of claim 16 wherein said DNA array is a polymorphism array.

18. The method of claim 17 wherein said polymorphism array is a SNP array.

19. The method of claim 1, further comprising quantitating said one or more nucleic acids.

20. The method of claim 19, wherein the amount of said one or more nucleic acids is indicative of transplant status or outcome.

21. The method of claim 20, wherein the amount of said one or more nucleic acids above a predetermined threshold value is indicative of a transplant status or outcome.

22. The method of claim 21, wherein said threshold is a normative value for clinically stable post-transplantation patients with no evidence of transplant rejection or other pathologies.

A0100

23. The method of claim 21, wherein there are different predetermined threshold values for different transplant outcomes or status.

24. The method of claim 20 wherein temporal differences in the amount of said one or more nucleic acids are indicative of a transplant status or outcome.

25. The method of claim 1, wherein said marker profile is determined by genotyping said transplant donor.

26. The method of claim 25, further comprising genotyping said subject receiving said transplant.

27. The method of claim 26, further comprising establishing a profile of markers, wherein said markers are distinguishable between said transplant donor and said subject receiving said transplant.

28. The method of any of the claims 25 to 27 wherein said genotyping is performed by a method selected from the group consisting of sequencing, nucleic acid array and PCR.

29. The method of claim 1 wherein said method has at least 56 % sensitivity.

30. The method of claim 1 wherein said method has at least 78 % sensitivity.

31. The method of claim 1 wherein said method has a specificity of about 70% to about 100%.

32. The method of claim 1 wherein said method has a specificity of about 80% to about 100%.

33. The method of claim 1 wherein said method has a specificity of about 90% to about 100%.

34. The method of claim 1 wherein said method has a specificity of about 100%.

35. A computer readable medium comprising:
        a set of instructions recorded thereon to cause a computer to perform the steps of:

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A0101

(i) receiving data from one or more nucleic acids detected in a sample from a subject who has received transplant from a donor, wherein said one or more nucleic acids are nucleic acids from said donor transplant, and wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile; and

(ii) diagnosing or predicting transplant status or outcome based on the presence or absence of said one or more nucleic acids.

A0102

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

77974 7590 08/23/2013	EXAMINER
Stanford University Office of Technology Licensing	BUNKER, AMY M
Bozicevic, Field & Francis LLP	

ART UNIT	PAPER NUMBER
1639	

1900 University Avenue
Suite 200
East Palo Alto, CA 94303

NOTIFICATION DATE	DELIVERY MODE
08/23/2013	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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PTOL-90A (Rev. 04/07)

A0103

Applicant-Initiated Interview Summary	Application No.	Applicant(s)
	13/508,318	QUAKE ET AL.
	Examiner	**Art Unit**
	AMY M. BUNKER	1639

All participants (applicant, applicant's representative, PTO personnel):

(1) *AMY M. BUNKER*.   (3)*Pamela Sherwood*.

(2) *Maria Leavitt*.   (4)*Kimberley Stopak and Lucia Muntean*.

Date of Interview: *16 August 2013*.

Type: ☒ Telephonic   ☐ Video Conference
          ☐ Personal [copy given to: ☐ applicant   ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes   ☒ No.
    If Yes, brief description: _____.

Issues Discussed   ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *36 and 39*.

Identification of prior art discussed: *Lo Yuk-Ming et al. (US2005/0282185) and Saint-Mezard et al. (WO2009/060035A1)*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The rejection of the claims under 35 U.S.C. 103(a) was discussed ==including, the broad scope of the independent claim, as well as, proposed limitations in the claimed methods to limit the scope of the claims in order to capture what the Applicant believes to be the substance and novelty of the invention .==

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Maria Leavitt/
Primary Examiner, Art Unit 1633

A0104

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

_____

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.
It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
– Application Number (Series Code and Serial Number)
– Name of applicant
– Name of examiner
– Date of interview
– Type of interview (telephonic, video-conference, or personal)
– Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
– An indication whether or not an exhibit was shown or a demonstration conducted
– An identification of the specific prior art discussed
–   An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
– The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.
A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.
Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

A0105

REMARKS

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are currently pending. Claims 1-35, 37, 38, 40, 43, 52, 55, 58-65, 68, and 69 are cancelled. Claims 36, 42, 44, 50 and 51 are currently amended. Support for the amended claims can be found in original claims 13, 14 and 29. No new matter has been added.

Interview Summary

Applicant's attorneys greatly appreciate the courtesy that was extended by Examiner Bunker and Supervisory Patent Examiner Leavitt during the telephonic interview conducted on August 16, 2013 with Pamela Sherwood, Kim Stopak and Lucia Muntean. During the interview, the scope of the obviousness rejection of record, particularly differences between prior art references (Lo Yuk-Ming *et al.* and Saint-Mezard *et al.*) and the claimed invention were discussed. The Office and Applicants discussed proposed limitations to the claimed methods to limit the scope of the claims in order to capture what the Applicant believed to be the substance and novelty of the invention.

35 U.S.C. § 112

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are rejected under 35 U.S.C. § 112, second paragraph (indefiniteness). To expedite prosecution and without conceding to the Examiner's rejections, Applicant has removed "derived from" from claims 36 and amended claim 36 to recite "wherein the one or more nucleic acids originated from the transplant from the donor." Applicant has also amended claims 42, 44 and 51 to delete "derived from the transplant from the donor" and to recite proper antecedent basis. Applicant has cancelled claim 43. Accordingly, the claims distinctly claim the subject matter which the applicant regards as the invention, and withdrawal of this rejection is respectfully requested.

Claim 36(c) is rejected under 35 U.S.C. § 112, second paragraph (indefiniteness). Applicant has amended claim 36(c) to recite proper antecedent basis. Accordingly, the claims distinctly claim the subject matter which the applicant regards as the invention, and withdrawal of this rejection is respectfully requested.

35 U.S.C. § 103

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Lo Yuk-Ming *et al.* (U.S. Patent Application No.

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A0106

20050282185, published December 22, 2005), and in view of Saint-Mezard *et al.* (International Patent Application No. WO2009060035A1, published May 14, 2009). To expedite prosecution and without conceding to the Examiner's rejections, Applicant has amended claim 36 to recite the following limitations: 1) circulating, cell-free nucleic acids; 2) detecting the one or more circulating, cell-free nucleic acid comprises sequencing the one or more circulating, cell-free nucleic acids; and 3) the sensitivity of the method is greater than 56%.

Neither Lo Yuk-Ming *et al.* nor Saint-Mezard *et al.*, either alone or in combination, teach, suggest or motivate one of ordinary skill in the art to make the claimed invention. As the Office admitted on page 7 of the Office Action, the combined references of Lo Yuk-Ming *et al.* and Saint-Mezard *et al.* do not teach where the sensitivity of the method is greater than 56%. In addition, neither Lo Yuk-Ming *et al.* and Saint-Mezard *et al.*, either alone or in combination, teach, suggest, or motivate one of ordinary skill in the art to detect circulating, cell-free nucleic acids originating from an organ from a donor by sequencing the circulating, cell-free nucleic acids.

On page 6 of the Office Action, the Office states that Lo Yuk-Ming *et al.* teach that "fetal DNA in maternal plasma was detected using direct sequencing; and that DNA sequencing on purified PCR products were analyzed using an ABI Prism 310 Genetic Analyzer." Applicants note that Lo Yuk-Ming's reference to sequencing pertains to the detection of fetal DNA in maternal plasma, but does not pertain to the detection of DNA originating from an organ from a donor. Furthermore, in reference to the use of sequencing to detect fetal DNA, Lo Yuk-Ming states in paragraph [0070] that "of these 8 informative cases, only a weak positive signal was observed in **one** of the 3rd trimester samples on direct sequencing." Thus, according to Lo Yuk-Ming, the sensitivity of the method is only 1 out of 8 or 12.5%. In paragraph [0071], Lo Yuk-Ming reasoned that "the weak signal in this single positive case and the low detection rate of the unmethylated fetal allele from maternal plasma might be due to the **low sensitivity of the direct sequencing method**. To enhance the sensitivity of detection, we employed a more sensitive primer extension assay to detect the unmethylated fetal allele from the MSP reaction products." Lo Yuk-Ming *et al.* does not teach, suggest, or motivate one of skill in the art to sequence DNA originating from a transplanted organ. In fact, Lo Yuk-Ming *et al.* teach away from the use of sequencing to detect DNA originating from a transplanted organ. Thus, due to the low sensitivity (12.5%) of the direct sequencing method to detect fetal DNA in maternal

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Atty Dkt. No.: STAN-706
USSN: 13/508,318

plasma, one of skill in the art would not have a reasonable expectation of success in detecting DNA originating from a transplanted organ by sequencing.

Thus, as neither Lo Yuk-Ming *et al.* nor Saint-Mezard *et al.*, either alone or in combination, teach, suggest or motivate one of ordinary skill in the art to make the claimed invention, Applicants respectfully request withdrawal of this rejection.
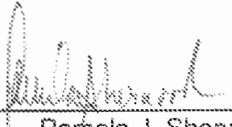
Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number STAN-706.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: October 10, 2013

By
Pamela J. Sherwood, Ph.D.
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A0108

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

77974   7590   11/14/2013
Stanford University Office of Technology Licensing
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1900 University Avenue
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EXAMINER
BUNKER, AMY M

ART UNIT	PAPER NUMBER
1639	

NOTIFICATION DATE	DELIVERY MODE
11/14/2013	ELECTRONICELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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zuehlke@bozpat.com
zizzo@bozpat.com

PTOL-90A (Rev. 04/07)

A0109

	Application No. 13/508,318	Applicant(s) QUAKE ET AL.
Office Action Summary	Examiner AMY M. BUNKER	Art Unit 1639	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *10 October 2013*.
    ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**.   2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *36,39,41,42,44-51,53,54,56,57,66 and 67* is/are pending in the application.
    5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *36,39,41,42,44-51,53,54,56,57,66 and 67* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined <u>allowable</u>, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
    a)☐ All   b)☐ Some *   c)☐ None of the:
       1.☐ Certified copies of the priority documents have been received.
       2.☐ Certified copies of the priority documents have been received in Application No. _____.
       3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
    * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☒ Notice of References Cited (PTO-892)

2)☐ Information Disclosure Statement(s) (PTO/SB/08)
     Paper No(s)/Mail Date _____.

3)☐ Interview Summary (PTO-413)
     Paper No(s)/Mail Date. _____ .

4)☐ Other: _____.

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The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office Action.

Status of Claims

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are currently pending. Claims 36, 42, 44, 50 and 51 have been amended by Applicants amendment filed on 10-10-2013. Claims 40, 43 and 58 have been canceled by Applicant's amendment filed on 10-10-2013.

Applicant's election *with traverse* of Group I, claims 36, 39-51, 53, 54, 56-58, 66 and 67, with traverse and the election of species: (A) single nucleotide polymorphisms (B) kidney transplant in the reply filed on March 21, 2013 was previously acknowledged.

No claims were withdrawn from consideration.

A complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

Accordingly, claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are under consideration to which the following grounds of rejection are applicable.

Priority

The present application is a 35 U.S.C. 371 national stage filing of International Application No. PCT/US2010/055604, filed on November 5, 2010, which claims the benefit of US Patent Application No. 61/280,674, filed on November 6, 2009.

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Interview Summary

The telephone interview between the Examiner, Primary Examiner Maria Leavitt, Pamela Sherwood, Kim Stopak and Lucia Muntean on August 16, 2013 discussing the scope of the obviousness rejection of record was previously acknowledged.

Withdrawn Rejections

Applicants' amendment and arguments filed April 16, 2013 are acknowledged and have been fully considered. The Examiner has re-weighed all the evidence of record. Any rejection and/or objection not specifically addressed below are herein withdrawn.

Claim Rejections - 35 USC § 112

The rejection of claims 36, 39-51, 53, 54, 56, 57, 66 and 67 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn, and the rejection of claim 58 is rendered moot, for the recitation of the term "derived from" due to Applicants amendment of claim 36 to replace the term with "wherein the one or more nucleic acids originated from the transplant donor" and Applicants cancellation of claim 58.

The rejection of claims 36, 39-51, 53, 54, 56, 57, 66 and 67 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn, and the rejection of claim 58 is rendered moot, for insufficient antecedent basis for the term "transplant status" due to Applicants amendment of claim 36 to recite proper antecedent basis and Applicants cancellation of claim 58.

Claim Rejections - 35 USC § 103

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The rejection of claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are under 35

U.S.C. 103(a) is withdrawn, and the rejection of claims 40, 43 and 58 are rendered moot, as

being unpatentable over Lo Yuk-Ming *et al*. (U.S. Patent Application No. 20050282185,

published December 22, 2005) in view of Saint-Mezard *et al*. (International Patent Application

No. WO2009060035A1, published May 14, 2009) due to Applicants amendment of the claims

and Applicants cancellation of claims 40, 43 and 58.

The combined references of Lo Yuk-Ming et al. and Saint-Mezard et al. have been
narrowed by reciting, "detecting the one or more circulating, cell-free nucleic acids from the
transplant of the donor" vs. "detecting one or more nucleic acids derived from the transplant
from the donor (previous claim 36) which detecting comprises sequencing (claim 43, now
canceled), wherein the sensitivity of the method is 56%.In view of the withdrawn rejection,
applicant's arguments are rendered moot.

New Objections/Rejections

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all

obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or
described as set forth in section 102 of this title, if the differences between the subject
matter sought to be patented and the prior art are such that the subject matter as a whole
would have been obvious at the time the invention was made to a person having ordinary
skill in the art to which said subject matter pertains. Patentability shall not be negatived
by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the

claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various

claims was commonly owned at the time any inventions covered therein were made absent any

evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out

the inventor and invention dates of each claim that was not commonly owned at the time a later

invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c)

and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

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     Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are rejected under 35 U.S.C. 103(a) as being obvious over Moreira et al. (Clinical Chemistry, 2009, 55(11), 1958-1966); in view of Lo Yuk-Ming *et al*. (U.S. Patent Application No. 20050282185, published December 22, 2005); and further in view of Baxter-Lowe et al. (Clinical Chemistry, 2006, 52(4), 559-561) as evidenced by Applied Biosystems (ABI Prism® 7000 Sequence Detection System, Assays-on-Demand Gene Expression Products Protocol, 2003, 1-40). **This is a new rejection necessitated by amendment of the claims in the response filed October 10, 2013.**

     Moreira et al. teach the use of total cell-free DNA (t-CF-DNA) and **donor-derived cell-free DNA** (ddCF-DNA) from **urine** and **plasma** as a rapid non-invasive **biomarker** of **rejection** and long-term graft function and survival in **renal transplant patients** (kidney transplant) (instant claims 36, 41, 56, 57, 66 and 67) (pg. 1958, column 1, Background, entire paragraph), such that plasma and urine samples from 100 renal **transplant recipients** were obtained 3 months after transplantation (providing a sample from a subject who has received a transplant) and tCF-DNA and ddCF-DNA were analyzed by **quantitative PCR** (multiplex reaction/real-time PCR) of the HBB and TSPY1 genes using a Prism® 7000 Sequence Detection System by Applied Biosystems (shotgun sequencing), where plasma tCF-DNA concentrations increased markedly during acute rejection (AR) episodes, often before clinical diagnosis, and returned to reference values after **anti-rejection treatment** (administering an immunosuppressive drug) (instant claims 36, 42, 44, 47, 48 and 51) (pg. 1958, column 1, Methods, entire paragraph & column 1, Results, lines 1-4). Moreira et al. also teach that a cut-off plasma of 12,000 genome equivalents/mL tCF-DNA concentration correctly classified AR and non-AR episodes in 86% of post-transplantation complications (diagnostic sensitivity, 89% and specificity, 85%) (sensitivity greater than 56%) (instant claim 36) (pg. 1958, column 1, Results, lines 4-8). Moreira et al. further teach that ddCF-DNA concentrations, up to 2000 genome equivalents/mL (detect at least ten different nucleic acids), were measured in women who had received a graft from a male donor and that ddCF-DNA was detected immediately after transplantation in patients without AR and infection, while plasma concentrations were undetectable within the first week after transplantation, such that patients who developed AR and graft infection showed a marked increase in the concentration of ddCF-DNA, which became undetectable after appropriate

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immunosuppressive treatment (pg. 1958, columns 1 & 2, Utility of ddCF-DNA, entire paragraph and pg. 1963, Fig 4.).

       Moreira et al. do not teach where the one or more nucleic acids derived from the donor are detected based on a marker profile comprising one or more genetic variations selected from those listed in instant claim 39 (instant claim 39), or where detecting comprises at least ten different nucleic acids (instant claim 46); or where the transplant status or outcome comprises modifying or maintaining an immunosuppressive regimen (instant claim 49); or where detecting comprises detecting genetic variations (instant claim 50); or where the method of claim 50 comprises genetic variations listed in instant claim 53 (instant claim 53); or where the method comprises at least one single nucleotide polymorphism (instant claim 54). Although Moreira et al. do not specifically teach where the multiplexed reaction occurs in a single container, the Prism® 7000 Sequence Detection System is designed with plates holding individual wells (single container) as evidenced by Applied Biosystems (pg. 5) (instant claim 45).

       The Lo Yuk-Ming et al. reference is relied upon for the reasons of record. Particularly, Lo Yuk-Ming et al. teach a method of differentiating DNA of an organ **donor** from **DNA** of an organ **recipient** using a **biological sample** such as plasma or serum (e.g., cell-free DNA) in order to **predict** the clinical progress of the transplantation recipient especially applied to **organ rejection** (paragraph [0030]), where DNA includes any sequence of more than one nucleotide such as polynucleotides, gene fragments and complete gene sequences, as well as, the study of **single nucleotide polymorphisms** (SNPs) (elected species), as well as, methylated and unmethylated alleles (e.g., genetic variations) (instant claims 39, 50, 53 and 54) (paragraphs [0012], lines 6-8, [0019] and Fig. 2). Lo Yuk-Ming et al. teach that the assay is applicable to the study of cellular chimerism following **solid organ** transplantation (e.g., liver, spleen, heart, pancreas, and kidneys), post-translational plasma DNA chimerism and urinary DNA chimerism. Lo Yuk-Ming et al. also teach SNPs in SEQ ID NOS: 1-11 (detecting at least ten different nucleic acid sequences) (instant claim 46) (pgs. 8-10, sequence listing).

       The combined references of Moreira et al. and Lo Yuk-Ming et al. do not teach where the transplant status or outcome comprises modifying or maintaining an immunosuppressive regimen (instant claim 49).

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   Baxter-Lowe et al. teach that one of the most promising areas of transplantation research is the discovery of biomarkers for rejection that are detectable in blood and urine, such that the development of non-invasive assays detecting molecular biomarkers for rejection by; (a) detecting a pre-rejection profile that will allow therapeutic interventions before rejection causes graft dysfunction, (b) improving the sensitivity and specificity of rejection diagnosis, (c) developing new classification systems for rejection that improve prognosis, and (d) providing information for designing individualized **immunosuppressive regimes** that could prevent rejection while minimizing drug toxicity (determining/modifying immunosuppressive regimen) (instant claim 49) (pg. 559, first full paragraph).

   In view of the teachings of Moreira et al., which exemplifies the use of total cell-free DNA (tCF-DNA) and donor-derived cell-free DNA (ddCF-DNA) from urine and plasma as a highly sensitive, rapid and non-invasive biomarker of rejection and long-term graft function and survival in renal transplant patients, where tCF-DNA and ddCF-DNA were analyzed by quantitative PCR; and in view of the teachings of Lo Yuk-Ming et al., which disclose a method of differentiating DNA of an organ donor from DNA of an organ recipient using a biological sample such as plasma or serum in order to predict the clinical progress of the transplantation recipient especially applied to solid organ rejection, where DNA includes any sequence of more than one nucleotide such as polynucleotides, gene fragments and complete gene sequences, as well as, the study of single nucleotide polymorphisms (SNPs), as well as, methylated and unmethylated alleles (e.g., genetic variations); and in view of the teachings of Baxter-Lowe et al., which describe the discovery of biomarkers for rejection that are detectable in blood and urine, such that the development of non-invasive assays detecting molecular biomarkers for rejection by detecting a pre-rejection profile that will allow therapeutic interventions before rejection causes graft dysfunction and providing information for designing individualized immunosuppressive regimes that could prevent rejection while minimizing drug toxicity; one of ordinary skill in the art at the time the invention was made would be motivated to use the variants taught by Lo Yuk-Ming et al. in the method taught by Moreira et al. to analyze donor-derived cell free DNA for the diagnosis and prognosis of graft rejection and, furthermore, to apply a pre-rejection profile for the development therapeutic intervention regimens as taught by Baxter-Lowe et al. for an efficient and non-invasive early detection of biomarkers of organ

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rejection and the development of personalized immunosuppressive regimens. In addition, one of ordinary skill in the art would have a reasonable expectation of success in using the genetic variants taught by Lo Yuk-Ming et al. in the method taught by Moreira et al. to detect biomarkers in urine and blood without having to conduct invasive biopsies in order to diagnosis transplant rejection and to allow for early immunosuppressive intervention as taught by Baxter-Lowe et al. This reasonable expectation of success would motivate one of ordinary skill in the art to modify the cited references.

The cited references teach the limitations of the claims and, therefore, the invention, as a whole, was *prima facie* obvious to one of ordinary skill in the art.

Conclusion

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are rejected.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to AMY M. BUNKER whose telephone number is (313) 446-4833. The examiner can normally be reached on 7:00am - 4:00pm.

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   If attempts to reach the examiner by telephone are unsuccessful, the examiner's
supervisor, Heather Calamita, can be reached on (571) 272-2876. The fax phone number for the
organization where this application or proceeding is assigned is (571) 273-8300.

   Information regarding the status of an application may be obtained from the Patent
Application Information Retrieval (PAIR) system. Status information for published applications
may be obtained from either Private PAIR or Public PAIR. Status information for unpublished
applications is available through Private PAIR only. For more information about the PAIR
system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR
system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would
like assistance from a USPTO Customer Service Representative or access to the automated
information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/AMY M BUNKER/
Examiner, Art Unit 1639

/Maria Leavitt/
Primary Examiner, Art Unit 1633

UNITED STATES PATENT AND TRADEMARK OFFICE

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NOTICE OF ALLOWANCE AND FEE(S) DUE

77974 7590 02/12/2014 Stanford University Office of Technology Licensing Bozicevic, Field & Francis LLP 1900 University Avenue Suite 200 East Palo Alto, CA 94303	**EXAMINER** BUNKER, AMY M

ART UNIT	PAPER NUMBER
1639	

DATE MAILED: 02/12/2014

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

TITLE OF INVENTION: Non-Invasive Diagnosis of Graft Rejection in Organ Transplant Patients

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0	$0	$480	05/12/2014

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. **SEE 37 CFR 1.313 AND MPEP 1308.**

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

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PTOL-85 (Rev. 02/11)

A0119

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: <u>Mail</u> Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or <u>Fax</u> (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

77974   7590   02/12/2014
Stanford University Office of Technology Licensing
Bozicevic, Field & Francis LLP
1900 University Avenue
Suite 200
East Palo Alto, CA 94303

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

	(Depositor's name)
	(Signature)
	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

TITLE OF INVENTION: Non-Invasive Diagnosis of Graft Rejection in Organ Transplant Patients

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$480	$0	$0	$480	05/12/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
BUNKER, AMY M	1639	506-007000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
❏ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
❏ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE   (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❏ Individual ❏ Corporation or other private group entity ❏ Government

4a. The following fee(s) are submitted:
❏ Issue Fee
❏ Publication Fee (No small entity discount permitted)
❏ Advance Order - # of Copies _____

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)
❏ A check is enclosed.
❏ Payment by credit card. Form PTO-2038 is attached.
❏ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
❏ Applicant certifying micro entity status. See 37 CFR 1.29

❏ Applicant asserting small entity status. See 37 CFR 1.27

❏ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____   Date _____

Typed or printed name _____   Registration No. _____

Page 2 of 3

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

77974 7590 02/12/2014	EXAMINER
Stanford University Office of Technology Licensing	BUNKER, AMY M

ART UNIT	PAPER NUMBER
1639	

Bozicevic, Field & Francis LLP
1900 University Avenue
Suite 200
East Palo Alto, CA 94303

DATE MAILED: 02/12/2014

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 02/11)

A0121

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

A0122

	Application No.	Applicant(s)	
Notice of Allowability	13/508,318	QUAKE ET AL.	
	Examiner	Art Unit	AIA (First Inventor to File) Status
	AMY M. BUNKER	1639	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the communication filed on 14 January 2014*.

  ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *36,39,41,44-49,51,54,56,57,66,67 and 70*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov .

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

  Certified copies:

  a) ☐ All   b) ☐ Some   *c) ☐ None of the:

    1. ☐ Certified copies of the priority documents have been received.

    2. ☐ Certified copies of the priority documents have been received in Application No. _____ .

    3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

  * Certified copies not received: _____ .

  Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application. **THIS THREE-MONTH PERIOD IS NOT EXTENDABLE**.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.

  ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

  Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)

2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____

3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material

4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .

5. ☒ Examiner's Amendment/Comment

6. ☒ Examiner's Statement of Reasons for Allowance

7. ☐ Other _____ .

/MARIA LEAVITT/
Primary Examiner, Art Unit 1633

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Art Unit: 1639

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Allowable Subject Matter

1.   This is in reply to papers filed on January 14, 2014. Claims 36, 39, 41, 44-51, 53, 54, 56, 57, 66 and 67 are now pending. Claim 36 has been amended by Applicant's amendment filed on 01-14-2014.

Therefore, claims 36, 39, 41, 44-51, 53, 54, 56, 57, 66 and 67 are under examination.

The examiner acknowledges receiving an executed Declaration under 37 C.F.R. § 1.132 signed by John F. Beausang on January 30, 2014 ("Beausang Decl."), and filed on January 31, 2014.

2.   Applicant's representative was contacted on January 28, 2014 to amend method claims 36, 41, and 51; add claim 70; and to cancel claims 50 and 53.

Authorization for the examiner's amendment was given in a telephone interview with Pamela Sherwood on January 30, 2014.

Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103, and 112.

3.   Accordingly, claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 are allowed.

Reasons for allowance

4.   The following is an examiner's statement of reasons for allowance:

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By amendment to the claims, Applicant has persuaded the Examiner that the prior art of
Moreira et al. (Clinical Chemistry, 2009, 55(11), 1958-1966); in view of Lo Yuk-Ming *et al.*
(U.S. Patent Application No. 20050282185, published December 22, 2005); and further in view
of Baxter-Lowe et al. (Clinical Chemistry, 2006, 52(4), 559-561) as evidenced by Applied
Biosystems (ABI Prism® 7000 Sequence Detection System, Assays-on-Demand Gene
Expression Products Protocol, 2003, 1-40), alone or in combination, do not teach or disclose a
method for detecting transplant rejection, graft dysfunction, or organ failure, particularly,
wherein the method comprises: obtaining a genotype of donor-specific polymorphisms or a
genotype of subject-specific polymorphisms, or obtaining both a genotype of donor-specific
polymorphisms and subject-specific polymorphisms to establish a polymorphism profile for
detecting donor cell-free nucleic acids, wherein at least one single nucleotide polymorphism
(SNP) is homozygous for the subject if the genotype comprises subject-specific polymorphisms
comprising SNPs; multiplex sequencing of the cell-free nucleic acids in the sample followed by
analysis of the sequencing results using the polymorphism profile to detect donor cell-free
nucleic acids and subject cell-free nucleic acids, wherein an increase in the quantity of the donor
cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ
failure, and wherein the sensitivity of the method is greater than 56% compared to sensitivity of
current surveillance methods for cardiac allograft vasculopathy (CAV).

The Examiner has also been persuaded by the Beausang Decl., indicating that the method
of claim 36 would work with genotype information from the donor, genotype information from
the transplant recipient or genotype information from both the donor and the transplant recipient,
where the information from the genotype data can be used to determine which SNPs in the donor
differ from the recipient by at least one allele, such that in the event data for only one genotype is
available (either donor or recipient) the genotype of the other can be inferred from publically
available databases (e.g.; dbSNP) that report the most likely allele frequencies in the population
at each SNP site.

Examiner's Amendment

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5.   In the claims:

Claims 50 and 53 have been cancelled.

Claim 70 has been added.

Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 have been rewritten as follows:

1-35.   (Cancelled)

36.   A method for detecting transplant rejection, graft dysfunction, or organ failure, the
       method comprising:
       (a) providing a sample comprising cell-free nucleic acids from a subject who has
            received a transplant from a donor;
       (b) obtaining a genotype of donor-specific polymorphisms or a genotype of
            subject-specific polymorphisms, or obtaining both a genotype of donor-specific
            polymorphisms and subject-specific polymorphisms, to establish a
            polymorphism profile for detecting donor cell-free nucleic acids, wherein at
            least one single nucleotide polymorphism (SNP) is homozygous for the subject
            if the genotype comprises subject-specific polymorphisms comprising SNPs;
       (c) multiplex sequencing of the cell-free nucleic acids in the sample followed by
            analysis of the sequencing results using the polymorphism profile to detect
            donor cell-free nucleic acids and subject cell-free nucleic acids; and
       (d) diagnosing, predicting, or monitoring a transplant status or outcome of the
            subject who has received the transplant by determining a quantity of the donor
            cell-free nucleic acids based on the detection of the donor cell-free nucleic
            acids and subject cell-free nucleic acids by the multiplexed sequencing,
            wherein an increase in the quantity of the donor cell-free nucleic acids over
            time is indicative of transplant rejection, graft dysfunction or organ failure, and

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wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).

37.   (Cancelled)

38.   (Cancelled)

39.   The method of claim 36, wherein the polymorphism profile comprises one or more genetic variations selected from single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, insertion elements, insertions, repeats, or deletions.

40.   (Cancelled)

41.   The method of claim 36, wherein the cell-free nucleic acids are deoxyribonucleic acid (DNA).

42.   (Cancelled)

43.   (Cancelled)

44.   The method of claim 36, wherein the multiplexed sequencing comprises shotgun sequencing.

45.   The method of claim 36, wherein the multiplexed sequencing occurs in a single container.

46.   The method of claim 36, wherein the multiplexed sequencing comprises sequencing at least ten different nucleic acids.

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47.   The method of claim 36 further comprising administering an immunosuppressive drug.

48.   The method of claim 36, wherein the diagnosing, predicting, or monitoring transplant status or outcome comprises treating a transplant rejection in a subject in need thereof.

49.   The method of claim 36, wherein the diagnosing, predicting, or monitoring transplant status or outcome comprises determining, modifying, or maintaining an immunosuppressive regimen.

50.   (Cancelled)

51.   The method of claim 36 further comprising conducting an assay selected from: digital polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), array, or any combination thereof.

52.   (Cancelled)

53.   (Cancelled)

54.   The method of claim 36, wherein the polymorphism profile comprises at least one single nucleotide polymorphism.

55.   (Cancelled)

56.   The method of claim 36, wherein the sample is blood or serum.

57.   The method of claim 36, wherein the sample is urine or stool.

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58-65. (Cancelled)

66.   The method of 36, wherein the transplant is selected from the group consisting of:
        kidney transplant, pancreas transplant, liver transplant, heart transplant, lung
        transplant, intestine transplant, pancreas after kidney transplant, and simultaneous
        pancreas-kidney transplant.

67.   The method of claim 36, wherein the transplant is a heart transplant or kidney
        transplant.

68.   (Cancelled)

69.   (Cancelled)

70.   The method of claim 36, wherein the cell-free nucleic acids are ribonucleic acid
(RNA).

        Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 are allowed.

        Any inquiry considered necessary by applicant must be submitted no later than the
payment of the issue fee and, to avoid processing delays, should preferably accompany the issue
fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for
Allowance."
        Any inquiry concerning this communication or earlier communications from the
examiner should be directed to AMY M. BUNKER whose telephone number is (313) 446-4833.
The examiner can normally be reached on Monday through Friday 7:00am to 4:00pm EST.

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     If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Heather Calamita can be reached on (571) 272-2876. The fax phone number for the organization where this application or proceeding is assigned is (571) 273-8300.

     Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/AMY M BUNKER/
Examiner, Art Unit 1639

/MARIA LEAVITT/
Primary Examiner, Art Unit 1633

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1

1   IN THE UNITED STATES DISTRICT COURT

2   FOR THE DISTRICT OF DELAWARE

3

4 CAREDX, INC.,   )
)
5   Plaintiff,   )
)
6   vs.   )No. 19-cv-662 (CFC)(CJB)
)
7 NATERA, INC.,   )
)
8   Defendant.   )
   _____)

9

10

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12

13

14

15   * * * CONFIDENTIAL – ATTORNEYS' EYES ONLY * * *

16   REMOTE VIDEOTAPED DEPOSITION OF

17   UWE CHRISTIANS, M.D., Ph.D.

18   MONDAY, NOVEMBER 9, 2020

19

20

21

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23

24

25 Reported in Stenotype by:
    Cody R. Knacke, CSR No. 13691
    Job No.: 2020-92907

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Attorneys Eyes OnlyUwe Christians - November 09, 2020

2

1   REMOTE VIDEOTAPED DEPOSITION OF

2 UWE CHRISTIANS, M.D., Ph.D., taken before Cody R.

3 Knacke, CSR No. 13691, a Certified Shorthand Reporter

4 for the State of California, with principal office in

5 the County of Los Angeles, commencing on Monday,

6 November 9, 2020, at 8:03 a.m., Mountain Time.

7

8 (All Appearances Via Videoconference.)

9 APPEARANCES OF COUNSEL:

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16
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21   Also Present:

22   Chris Weiss Calhoon, Videographer

23

24

25

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1   MS. ACHTERHOF: Objection.

2   THE WITNESS: Is that what you're showing me

3   right now?

4   BY MR. REINES:

5   Q.   No.

6   A.   Okay.

7   Q.   Have you seen evidence in this case that the

8   study design differences between the Bloom study and

9   the Natera study were fundamental?

10   A.   There are some design differences, but I

11 wouldn't say they're fundamental.

12   Q.   So you disagree that they're fundamental, is

13 that correct, that the differences --

14   A.   I disagree. I disagree, with the

15 fundamental.

16   Q.   Understood.

17   You'll agree that there are study design

18 differences, though, between the Natera study and the

19 Bloom study; correct?

20   A.   Yes.

21   Q.   And Dr. Sarwal stated that she -- in

22 comparing the two studies, she wouldn't know how to

23 compensate for those differences.

24   Do you see that?

25   MS. ACHTERHOF: Misstatement of the

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1   transcript.

2   THE WITNESS: To be honest, I think this

3   question is -- I -- I have no idea where this

4   question is coming from. And I completely agree with

5   her answer. I also don't know how you would

6   compensate for that.

7   BY MR. REINES:

8   Q.   So in terms of --

9   A.   Is there a mathematical algorithm or

10 something there? I don't know.

11   Q.   In -- in comparing the results of the Bloom

12 study to the Natera study, you wouldn't know how to

13 compensate for the study design differences; correct?

14   A.   There is no need to compensate for the study

15 design differences. Your question is completely

16 baseless.

17   Q.   It's absolutely and completely baseless. Is

18 that your position?

19   A.   It's absolutely baseless, yes.

20   Q.   And because there's no need at all to

21 compensate for the study design differences between

22 the Bloom and the Natera studies, correct, according

23 to you?

24   A.   I think there is no need for it, and there

25 is also no way to do it. But, I mean, I thought that

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1 carrying the extract would compensate for that, and

2 this was a terrible idea.

3   Q.   And so you believe from the Bloom study and

4 the Natera study could be compared head to head;

5 correct?

6   MS. ACHTERHOF: Objection.

7   THE WITNESS: I think it can be compared.

8 In terms of head to head, we can have a discussion

9 about that. That's in my -- that's in my expert

10 report, that I don't agree with a head to head.

11 BY MR. REINES:

12   Q.   Why is it that the Natera study and the

13 Bloom study can't be compared head to head?

14   A.   Because they're not the same study. So by

15 definition, if you do a head to head in an analytical

16 study, this means that you pick the sample, you split

17 it, you send it, you measure it with one test, and

18 the other half you measure with the other test.

19   That is not what has happened here. And

20 that's also something that, to the best of my

21 knowledge, Natera has never claimed. In fact, when

22 you look at some of the Natera documents, it says

23 that it was a comparison between separate studies,

24 and I can 100 percent agree to that.

25   Q.   Yeah, there is -- my -- that's not

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1   responsive in your answering my question.

2   My question is just, what are the dangers of

3   trying to compare head-to-head studies that have

4   different study designs?

5   MS. ACHTERHOF: Objection.

6   THE WITNESS: I mean, whenever you compare

7   studies that are separate studies, there is always

8   certain, let's say, drawbacks by doing this. And

9   so -- but this doesn't mean that studies can't be

10 compared.

11 BY MR. REINES:

12   Q.   No. My question was -- so let me go back.

13   Do you have an opinion in your report that

14 you can't compare the Bloom study and the Natera

15 study head to head? Correct?

16   A.   I have an opinion that the Bloom study and

17 the -- the Sarwal study, Sigdel study can be

18 compared. I would just not call it a head-to-head

19 comparison.

20   Q.   And what are the -- what do you think are

21 the minimum requirements in order to perform a

22 head-to-head comparison of two diagnostic tests?

23 What safeguards do you need?

24   A.   That needs to be decided on a case-to -- on

25 a case-to-case basis, but in this specific case here,

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1   A.   Yes, I did.

2   Q.   And you took them into account in preparing

3   your opinion?

4   A.   Yes, I did.

5   Q.   And do you know who Dr. Gauthier is?

6   A.   Yes, I do.

7   Q.   And you understand he's the medical director

8   of the organ health division of Natera?

9   A.   That's my understanding.

10   Q.   And Dr. Gauthier testified that the study

11   design of the Bloom study is fundamentally different

12   from the study design for the Sarwal study.

13   Do you see that?

14   A.   I see that.

15   MS. ACHTERHOF: Objection.

16   BY MR. REINES:

17   Q.   And you're saying you disagree with

18   Dr. Gauthier, medical director of organ health for

19   Natera, about this?

20   MS. ACHTERHOF: Objection.

21   THE WITNESS: Disagree.

22   BY MR. REINES:

23   Q.   Did you attempt to talk to Dr. Gauthier to

24   understand why he would say something that he thinks

25   inaccurate?

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1   A.   I told you I didn't talk to anybody at

2   Natera.

3   Q.   And who do you think's more familiar with

4   the Natera study, you or Dr. Gauthier?

5   MS. ACHTERHOF: Objection.

6   THE WITNESS: I can't answer this. I don't

7   know how far he was involved in the study. It's the

8   training field for Natera; so I'm not sure how much

9   he was involved in the details.

10 BY MR. REINES:

11   Q.   And what are the dangers of comparing the

12 results of studies with different design, study

13 designs, and presenting that as head to head?

14   MS. ACHTERHOF: Objection to form.

15   THE WITNESS: I mean, this comes back to the

16 head to head. I mean, nobody presented this as head

17 to head. Nobody can claim that here.

18   But the dangers of different study designs,

19 one of the things you have to look at is to which

20 extent are these studies comparable, and then one has

21 to do a gap analysis and has to see what are the

22 risks of comparing these studies.

23   I mean, comparing studies with different

24 designs is something that is done in medicine all the

25 time, and every review article does this. And

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1   there's even a whole science about this when you look

2   at the Cochrane Database and these kinds of things.

3   And this is something that fundamentally

4   drives medicine. So comparing data from studies with

5   different designs is nothing that is unusual or

6   unscientific.

7   MR. REINES: Move to strike that as

8   nonresponsive to my question.

9   BY MR. REINES:

10   Q.   And I ask the -- remind the witness again to

11   be responsive to my question.

12   Okay. Let --

13   MS. ACHTERHOF: For the record, I disagree.

14   It was responsive to the extent it could be.

15   MR. REINES: Okay. I'd ask the reader to go

16   up and read the question and answer and then consider

17   the comments from Natera.

18   And, by the way, I think encouraging

19   nonresponsive answers is improper.

20   MS. ACHTERHOF: I'm not encouraging

21   nonresponsive answers. I just disagreed with your

22   assessment of his answer.

23   MR. REINES: Right, that's what I'm saying.

24   And if the reader will go up and read that question

25   and answer and see what Ms. Achterhof is reinforcing

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 1   the witness to do, they can make their own judgment.

 2   It's just the last two or three pages of this
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 3   transcript.

 4   BY MR. REINES:

 5       Q.    I'd like to direct you to page 65 of the

 6   Billings transcript, which I believe is Exhibit 4.

 7           MR. MAGEE:  That's D, as in dog, -2.  We

 8   should have that open as a tab already.

 9           MR. REINES:  Oh, so is that --

10           MS. ACHTERHOF:  Look on the top.

11           MR. REINES:  Yeah.  I don't see it.

12           THE VIDEOGRAPHER:  Which tab do you want?

13           MR. REINES:  Oh, there you go.  It's

14   Exhibit 2, the Billings transcript at -- let's start

15   with 65, page 65, line 13 through 19.

16   BY MR. REINES:

17       Q.    Okay.  And this is from the chief medical

18   officer of Natera, Dr. Billings.

19           And you saw this testimony when -- when you

20   were preparing your opinions in this case; is that

21   correct?

22       A.    Yes.

23       Q.    And you considered this specific testimony
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24   when you prepared your opinion in this case?

25       A.    Yes, I did.
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1   MS. ACHTERHOF: Yes. I just have a couple.

2   EXAMINATION

3   BY MS. ACHTERHOF:

4   Q.   So, Dr. Christians, you testified earlier

5   that you believe that there are no tests for organ

6   rejection in the kidney area.

7   Would you please explain that statement.

8   A.   Yeah. So what I meant by this was that

9   there is no test that on its own tells me if there is

10 rejection or not. So there are many tests that can

11 indicate organ rejection, but you have to look at the

12 whole picture.

13   So it's not like -- I don't know -- like --

14 let's say, a test like troponin, where if you say,

15 well, if it's above a certain limit the patient has a

16 heart attack or not. So there's nothing like this

17 for -- for kidney transplant.

18   So it's mostly still guesswork. So the holy

19 grail of transplant nephrology would be to have an

20 assay which says, okay, you have a rejection, or you

21 don't.

22   So the only thing we have at the moment is

23 really assays that tell us when to procure a biopsy,

24 and then the biopsy is the ultimate gold standard,

25 which tells us if there is a rejection going on or

A0141

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1   not.

2   Q.   Earlier you testified regarding the

3   suitability of Prospera for use with children under

4   the age of 18.

5   Is there any way, in your opinion, that

6   Prospera could be suitable for use with children

7   under the age of 18?

8   A.   Well, first of all --

9   MR. REINES: Object to form.

10   THE WITNESS: -- I believe -- okay. First

11   of all, I believe Prospera is useful for children. I

12   mean, cell-free DNA in general would be useful for

13   children. The question is, is the data that Natera

14   has produced for the children useful in any way for

15   pediatric transplantation.

16   I have not seen any indication that actually

17   Natera has thought of it, but what you can do is,

18   based on the clinical laboratory stance as to

19   guidelines, you can analyze those 40 samples,

20   calculate the 95 percent confidence intervals with

21   this, and the upper 25 percent confidence interval

22   then would be your cutoff value.

23   So to define clinical cutoff values based on

24   stable patients or on healthy individuals is routine

25   and completely acceptable.

A0142

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1   So what they could do is, they could use

2   these 40 samples and define this cutoff for children

3   and then in the next study try to see if this cutoff

4   works.

5   BY MS. ACHTERHOF:

6   Q.   Just about there.

7   So, Dr. Christians, earlier in your

8   testimony you were asked whether the Natera marketing

9   literature showing Prospera outperforming AlloSure

10 is -- whether that is something that would be of

11 interest to you as a clinician trying to evaluate the

12 different tests.

13   Do you recall that question?

14   A.   Yes.

15   Q.   And your answer was:

16   "You know, to be honest, no.

17   And I will explain it to you."

18   Would you please provide your explanation

19 for that answer?

20   A.   Yes.

21   So, I mean, marketing literature for me, and

22 I can also speak for many of my colleagues that I

23 know, is to pique our interest. So if I see this

24 marketing literature, and I see this, and I say, "Oh,

25 this is interesting. Maybe this could be something,"

A0143

Attorneys Eyes OnlyUwe Christians - November 09, 2020

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 1              DEPOSITION OFFICER'S CERTIFICATE

 2

 3   COUNTY OF LOS ANGELES,    )

 4   STATE OF CALIFORNIA,      )

 5

 6          I, Cody R. Knacke, hereby certify:

 7          I am a duly-qualified Certified Shorthand

 8   Reporter in and for the State of California, holder

 9   of Certificate Number CSR 13691, issued by the Court

10   Reporters Board of California and which is in full

11   force and effect.  (Fed. R. Civ. P. 28(a)).

12          I am authorized to administer oaths or

13   affirmations pursuant to California Code of Civil

14   Procedure, Section 2093(b) and prior to being

15   examined, the witness was first duly sworn by me.

16   (Fed. R. Civ. P. 28(a), 30(f)(1)).

17          I am not a relative or employee or attorney

18   or counsel of any of the parties, nor am I a relative

19   or employee of such attorney or counsel, nor am I

20   financially interested in this action.  (Fed. R. Civ.

21   P. 28).

22          I am the deposition officer that

23   stenographically recorded the testimony in the

24   foregoing deposition and the foregoing transcript is

25   a true record of the testimony given by the witness.
```

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Attorneys Eyes OnlyUwe Christians - November 09, 2020

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1   (Fed. R. Civ. P. 30(f)(1)).

2   Before the completion of the deposition,

3   review of the transcript [X] was [   ] was not

4   requested. If requested, any changes made by the

5   deponent (and provided to the reporter) during the

6   period allowed, are appended hereto. (Fed. R. Civ.

7   P. 30(e)).

8   In witness whereof, I have hereunto set my

9   hand this day: _____, 2020.

10

11

12

13

14

15   _____
     CODY R. KNACKE, CSR No. 13691

16

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A0145

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 1                  IN THE UNITED STATES DISTRICT COURT

 2                    FOR THE DISTRICT OF DELAWARE

 3

 4       CAREDX, INC. and THE BOARD OF

         TRUSTEES OF THE LELAND

 5       STANFORD JUNIOR UNIVERSITY,

 6              Plaintiffs,

 7          vs.                        C.A. No. 19-567-CFC-CJB

 8       NATERA, INC.,

 9              Defendant.
         _____/

10

         CAREDX, INC.,

11

12              Plaintiffs,

         vs.                        C.A. No. 19-1804-CFC-CJB

13       EUROFINS VIRACOR,

14              Defendant,

15       THE BOARD OF TRUSTEES OF

         THE LELAND STANFORD JUNIOR

16       UNIVERSITY,

17              Nominal Defendant.
         _____/

18

19           DEPOSITION OF UWE CHRISTIANS, M.D., Ph.D.

20          appearing remotely at Castle Rock, Colorado

21                  Monday, March 29, 2021

22

         Reported by:

23       Natalie Y. Botelho
         CSR No. 9897

24

25       Job No. 4517676


                                              Page 1
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      Goodwin Procter
23
              LUKE BURTON, Natera (in-house)
24
              JILL WARREN, Videographer
25
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Page 3

1	that leads to the transplant. These are usually	09:33:25
2	very sick patients, so they need all kinds of	09:33:29
3	different diagnostic and kind of different care	09:33:33
4	which is not necessarily only focused on the	09:33:36
5	transplant organ itself.	09:33:38
6	Q. Okay. So a person of ordinary skill in	09:33:39
7	the art would have broader understanding of the	09:33:41
8	science related to medical diagnostics beyond the	09:33:45
9	specifics of organ transplant; is that what you're	09:33:49
10	saying?	09:33:54
11	MR. DeJONG: Objection to form.	09:33:55
12	THE WITNESS: Yes.	09:33:57
13	MR. WALTER: Q. Okay. And what skills	09:33:59
14	would that person have related to medical	09:34:01
15	diagnostics?	09:34:06
16	MS. HABERNY: Objection to form.	09:34:07
17	THE WITNESS: I mean, my definition is	09:34:10
18	that this person, for example, would be -- have an	09:34:12
19	M.D., and I mean, those are skills that are taught	09:34:16
20	in medical school, how to monitor and diagnose a	09:34:19
21	patient, and so this basic knowledge is necessary	09:34:22
22	for also managing a transplant patient.	09:34:27
23	MR. WALTER: Q. Okay. Well, let me ask	09:34:31
24	something more specific. Would a person of ordinary	09:34:37
25	skill in the art with at least three years'	09:34:39

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1	experience in medical diagnostics have an	09:34:42
2	understanding of statistics related to diagnostic	09:34:46
3	testing?	09:34:50
4	MS. HABERNY: Objection to form.	09:34:51
5	MR. DeJONG: Objection.	09:34:52
6	THE WITNESS: Well, a person of skill in	09:34:54
7	the art would have basic understanding how to	09:34:56
8	develop an assay, how to validate an assay, and of	09:34:59
9	course this person would also be familiar with the	09:35:02
10	statistics that's required for developing/validating	09:35:05
11	an assay and also to clinically validate an assay.	09:35:09
12	MR. WALTER: Q. So let's talk about some	09:35:13
13	of those statistics. One of those statistics is	09:35:15
14	sensitivity, correct?	09:35:17
15	A. Sensitivity is not statistics.	09:35:20
16	Q. It's not a statistic. What would you	09:35:23
17	characterize sensitivity as, then?	09:35:25
18	A. It's a performance parameter.	09:35:27
19	Q. Okay. So would someone with skill in the	09:35:29
20	art in the field of the '652 and '607 patents have	09:35:33
21	an understanding of the performance parameters that	09:35:40
22	are relevant to medical diagnostics?	09:35:43
23	MR. DeJONG: Objection to form.	09:35:48
24	MS. HABERNY: Objection to form.	09:35:50
25	THE WITNESS: Yes, of course. I mean,	09:35:51

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1   that's what you get taught in medical school.   09:35:52

2   MR. WALTER: Q. And you wouldn't   09:35:54

3   characterize that as a statistic?   09:35:55

4   A.   No.   09:35:57

5   Q.   Okay. So a person of ordinary skill in   09:35:58

6   the art of the '652 and '607 patents would have an   09:36:09

7   understanding of the sensitivity performance   09:36:13

8   parameter and what that means?   09:36:16

9   MR. DeJONG: Objection to form.   09:36:19

10   THE WITNESS: Yes, a person of skill in   09:36:21

11   the art would know what the sensitivity is.   09:36:23

12   MR. WALTER: Q. Okay. And they would   09:36:25

13   also have an understanding of what specificity is   09:36:26

14   and what that performance parameter means?   09:36:28

15   A.   Yes, they would.   09:36:32

16   Q.   Okay. Now, would the person of ordinary   09:36:33

17   skill in the art be familiar with the use of   09:36:36

18   thresholds or cutoffs in diagnostic testing?   09:36:41

19   MS. HABERNY: Objection to form.   09:36:45

20   THE WITNESS: Well, always -- yes, they   09:36:49

21   would know it because they're using them.   09:36:51

22   MR. WALTER: Q. Okay. And what are they?   09:36:53

23   What are cutoffs in diagnostic testing?   09:36:55

24   A.   Well, usually a cutoff is a value that   09:37:00

25   discriminates between the person having a certain   09:37:05

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A0150

1	condition or not having a certain condition.	09:37:09
2	Q. And how are cutoffs determined in	09:37:12
3	diagnostic testing?	09:37:20
4	MR. DeJONG: Objection to form.	09:37:24
5	THE WITNESS: First of all, it depends on	09:37:26
6	the parameter you're looking at. This depends on	09:37:28
7	the test you're looking at. And there are several	09:37:31
8	ways of determining cutoffs. There's really no	09:37:35
9	general answer for your question.	09:37:40
10	MR. WALTER: Q. What are some of the ways	09:37:41
11	of determining cutoffs that you're aware of?	09:37:42
12	A. Well, I mean, one of the ways to determine	09:37:46
13	a cutoff would be a receiver operating curve.	09:37:49
14	Q. Are there other ways that you're aware of?	09:37:55
15	A. Yes, you can determine a cutoff by simple	09:37:57
16	distribution statistics.	09:38:01
17	Q. Okay. What do you mean by that?	09:38:02
18	A. You can, for example, determine a cutoff	09:38:06
19	as a 95% confidence interval of a healthy	09:38:08
20	population. There's regulatory guidance as how to	09:38:12
21	determine cutoffs.	09:38:18
22	Q. And the person of ordinary -- I'm sorry.	09:38:19
23	Go ahead. Please finish.	09:38:21
24	A. Thank you. You would follow these	09:38:23
25	guidances because if you find a cutoff in which	09:38:24

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1	medical decisions are made, you have to be in	09:38:28
2	compliance with regulatory guidelines.	09:38:31
3	Q. Okay. And the person of ordinary skill in	09:38:33
4	the art would have been aware of those regulatory	09:38:35
5	guidelines?	09:38:37
6	MR. DeJONG: Objection to form.	09:38:38
7	THE WITNESS: Yes.	09:38:41
8	MR. WALTER: Q. Okay. And they would	09:38:44
9	have known the receiver operating curve and the	09:38:45
10	confidence interval method of determining the cutoff	09:38:48
11	that you describe?	09:38:51
12	MS. HABERNY: Objection to form.	09:38:52
13	THE WITNESS: They would know that this	09:38:53
14	would be one way of doing it.	09:38:55
15	MR. WALTER: Q. And there's probably	09:38:56
16	others that they would have been aware of as well,	09:38:57
17	right?	09:38:59
18	A. Yes.	09:39:01
19	Q. Okay. What are some of the others they	09:39:02
20	would have been aware of?	09:39:04
21	A. As I said, the -- I mean, there's also	09:39:06
22	empirical ways of doing it, but as I said, the most	09:39:11
23	important ways of doing it is receiver operating	09:39:13
24	curves, distribution statistics. There are other	09:39:18
25	more complex statistical ways of determining	09:39:21

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1	cutoffs.	09:39:24
2	Q. What are the empirical ways that you just	09:39:26
3	mentioned?	09:39:29
4	A. Well, I mean, if you define a cutoff, for	09:39:31
5	example, based on a receiver operating curve, then	09:39:33
6	you would have to decide which is the cutoff that	09:39:38
7	makes statistically the most sense. That means	09:39:42
8	which balances the sensitivity and specificity in a	09:39:44
9	way that it gives you the best accuracy of the assay	09:39:48
10	in the clinic. And the cutoff also always depends	09:39:52
11	on what you're interested in achieving with the	09:39:59
12	assay.	09:40:03
13	Q. And the person of ordinary skill in the	09:40:09
14	art would know how to do that?	09:40:11
15	MR. DeJONG: Objection to form.	09:40:13
16	THE WITNESS: They would definitely know	09:40:18
17	what the options are, yes.	09:40:20
18	MR. WALTER: Q. All right. Now, let me	09:40:26
19	ask you some basic questions about donor-derived	09:40:30
20	cell-free DNA in the context of organ transplant.	09:40:33
21	The person of ordinary skill in the art in 2009	09:40:37
22	would have been aware of the phenomenon of	09:40:41
23	donor-derived cell-free DNA in the context of organ	09:40:44
24	transplant; is that right?	09:40:47
25	MS. HABERNY: Objection to scope.	09:40:49

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1	THE WITNESS: Yes.	09:40:53
2	MR. WALTER: Q. Okay. And they would	09:40:55
3	have been aware -- would they have been aware that	09:40:59
4	during organ rejection, the concentration of	09:41:03
5	donor-derived cell-free DNA increases?	09:41:07
6	MS. HABERNY: Objection to scope.	09:41:10
7	THE WITNESS: Yes.	09:41:14
8	MR. WALTER: Q. Okay. And would the	09:41:14
9	person of ordinary skill in the art have been aware	09:41:18
10	that the increase in the amount of donor-derived	09:41:20
11	cell-free DNA may depend on the type of organ	09:41:27
12	transplant that's at issue?	09:41:30
13	A. Depends on the organ transplant that's in	09:41:37
14	issue. I think yes, he would.	09:41:41
15	Q. Okay. All right. Now let's consider the	09:41:44
16	context of organ transplant rejection where we're	09:41:51
17	trying to assess rejection based on the percentage	09:41:58
18	of donor-derived DNA in an organ recipient's	09:42:01
19	bloodstream. Okay? Do you have that in mind?	09:42:04
20	A. Yes.	09:42:08
21	Q. Okay. Now, the person of ordinary skill	09:42:09
22	in the art, to determine whether there's rejection,	09:42:11
23	wouldn't pick a cutoff at random, right?	09:42:15
24	MR. DeJONG: Objection to form.	09:42:20
25	THE WITNESS: No.	09:42:24

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 1    100%, but you would have a specificity that would      09:43:33

 2    make the test useless, right?                          09:43:36

 3            MR. DeJONG:  Objection to form.                 09:43:39

 4            THE WITNESS:  Yes, would be useless.            09:43:41

 5            MR. WALTER:  Q.  Okay.  Now, the person of      09:43:44

 6    ordinary skill in the art wouldn't split the           09:43:47

 7    difference and pick 50% either, would they?            09:43:49

 8            MR. DeJONG:  Objection to form.                 09:43:52

 9            THE WITNESS:  That is a question I don't        09:43:57

10    know because that depends on the specificity of the    09:43:58

11    test.  If the specificity is very high, then 50% may   09:44:02

12    still be of clinical value.                            09:44:07

13            MR. WALTER:  Q.  How would someone of           09:44:10

14    skill in the art go about picking the appropriate      09:44:11

15    threshold to use for a organ rejection assay based     09:44:14

16    on the percentage of donor-derived cell-free DNA?      09:44:19

17    A.       Well, you have to look at what donor --       09:44:24

18    cell-free donor -- cell-free donor-derived DNA for     09:44:29

19    what the assay is used for.  We don't know that        09:44:34

20    really yet.  I mean, if it's used as a triage assay,   09:44:38

21    so basically as a monitoring assay, that then would    09:44:44

22    trigger, for example, a biopsy.  Then one would pick   09:44:47

23    something that has a high sensitivity and would        09:44:52

24    accept a low specificity.                              09:44:54

25            If it would be used for making the             09:44:56
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1	diagnosis, then it's probably better to pick	09:44:59
2	something with a lower sensitivity and a very high	09:45:02
3	specificity. So it really depends on what you want	09:45:05
4	to achieve for the assay.	09:45:07
5	Q. And the person of ordinary skill in the	09:45:10
6	art would know how to select the cutoffs based on	09:45:11
7	what he or she wants to achieve, correct?	09:45:13
8	MS. HABERNY: Objection to form.	09:45:16
9	MR. DeJONG: Objection to form.	09:45:17
10	THE WITNESS: Yeah, if this is within	09:45:22
11	the -- within a reasonable range, I mean, of course	09:45:24
12	you would pick the sensitivity or choose the cutoff	09:45:28
13	based on what gives you your best option in terms of	09:45:34
14	what you want to achieve.	09:45:38
15	MR. WALTER: Q. And a person of ordinary	09:45:40
16	skill in the art would know how to make that	09:45:41
17	selection, right?	09:45:43
18	MS. HABERNY: Objection to form.	09:45:44
19	MR. DeJONG: Objection to form.	09:45:45
20	THE WITNESS: Yes, because this is a	09:45:46
21	person who knows about transplantation, and to make	09:45:47
22	this decision, you need to have a lot of experience	09:45:51
23	in transplantation.	09:45:53
24	MR. WALTER: Q. Okay. And if the person	09:45:55
25	of ordinary skill in the art picked the threshold	09:45:56

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1	such that the sensitivity they were using was below	09:45:59
2	56%, then that particular test wouldn't satisfy the	09:46:03
3	claims, right?	09:46:08
4	MS. HABERNY: Objection to form.	09:46:09
5	MR. DeJONG: Objection to form.	09:46:09
6	THE WITNESS: You know, I can't tell you	09:46:13
7	this because the way the claim are written, I would	09:46:15
8	not be able to understand that.	09:46:21
9	MR. WALTER: Q. Okay. Well, let me ask	09:46:24
10	some -- I'll come back to that, then. Would the	09:46:25
11	person of ordinary skill in the art be able to	09:46:30
12	tailor the threshold they're using in their test for	09:46:33
13	different types of organs?	09:46:38
14	MR. DeJONG: Objection to form.	09:46:41
15	THE WITNESS: I mean, yes, for different	09:46:45
16	types of organs, you will need different -- you	09:46:46
17	probably would pick a different threshold, and this	09:46:49
18	also simply has to do with the fact that the results	09:46:55
19	of the tests will be -- will be different.	09:46:57
20	MR. WALTER: Q. And a person of ordinary	09:47:01
21	skill in the art would know that?	09:47:02
22	MR. DeJONG: Objection; form.	09:47:04
23	MS. HABERNY: Objection; form.	09:47:04
24	THE WITNESS: Yes, person of skill in the	09:47:08
25	art would definitely be aware of this, yes.	09:47:09

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1	MR. WALTER: Q. And a person of ordinary	09:47:12
2	skill in the art would be able to tailor the cutoffs	09:47:13
3	that he or she might use depending on the timing of	09:47:19
4	when the assay is done post organ transplant?	09:47:23
5	MR. DeJONG: Objection to form.	09:47:27
6	MS. HABERNY: Objection to form.	09:47:28
7	THE WITNESS: Yeah, I mean, if that's	09:47:35
8	what -- if this is what the intention of the assay	09:47:38
9	is and this is what the person is interested in	09:47:40
10	terms of the diagnostic performance, then this would	09:47:43
11	be a factor to consider.	09:47:46
12	MR. WALTER: Q. Okay. And the person of	09:47:48
13	ordinary skill in the art would be able to tailor	09:47:50
14	those thresholds depending on the timing of when the	09:47:51
15	assay was done, right?	09:47:55
16	MS. HABERNY: Objection.	09:47:57
17	MR. DeJONG: Objection to form.	09:47:58
18	THE WITNESS: If -- yes, I mean, if you	09:48:02
19	have an assay that you want to use early after	09:48:03
20	transplantation, then the cutoffs would be different	09:48:06
21	than later, after transplantation.	09:48:08
22	MR. WALTER: Q. And how would the person	09:48:10
23	of ordinary skill in the art go about -- let's go	09:48:11
24	back to the situation of different organs. How	09:48:14
25	would the person of ordinary skill in the art go	09:48:16

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1	about tailoring the thresholds for the different	09:48:18
2	types of organ transplant?	09:48:21
3	A. That is a very general question that I	09:48:27
4	can't answer. I mean, this depends on the organ.	09:48:28
5	That depends on, you know, which disease mechanism	09:48:31
6	the person of skill in the art wants to diagnose. I	09:48:37
7	can't tell you this. This is -- you have to be a	09:48:42
8	lot more specific.	09:48:45
9	Q. Okay. Let's take a look at the document	09:48:54
10	that I've marked as Exhibit 2, which is the '652	09:48:56
11	patent. Do you have Exhibit 2, Dr. Christians?	09:48:58
12	A. Yes, I do.	09:49:02
13	Q. All right. Let's take a look at claim 1.	09:49:16
14	And you focus on the last clause of the claim, which	09:49:31
15	reads "written sensitivity of the method is greater	09:49:34
16	than 56% compared to sensitivity of current	09:49:36
17	surveillance methods for cardiac allograft	09:49:41
18	vasculopathy (CAV)." Do you see that?	09:49:45
19	A. Yes.	09:49:48
20	Q. Okay. Now I want to ask you a question:	09:49:48
21	If the claim simply read and -- let me try to make	09:49:50
22	the question clearer. If the claim simply read	09:49:57
23	"wherein sensitivity of the method is greater than	09:50:00
24	56%," would you contend that the claims are	09:50:04
25	indefinite?	09:50:07

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 1              MS. HABERNY:  Objection to form.           09:59:29

 2              MR. DeJONG:  Objection to form.            09:59:29

 3              THE WITNESS:  You have to define what kind 09:59:30

 4     of an assay.  I mean, if it's a predictive assay,  09:59:31

 5     diagnostic assay, monitoring assay.  We have many  09:59:34

 6     assays that look at all kinds of different aspects 09:59:37

 7     of rejection.                                      09:59:41

 8              For example, if you look at an assay to    09:59:42

 9     look at donor-specific antibodies, it clearly shows 09:59:44

10     that the patient is at risk and probably may need  09:59:49

11     protocol biopsies to make sure that there's no     09:59:53

12     rejection going on, but I mean, there's lots of    09:59:58

13     assays.  Could be as simple as measuring creatinine. 10:00:01

14     I don't know.  You have to tell me exactly what    10:00:05

15     assay you're talking about.                        10:00:10

16              MR. WALTER:  Q.  And then going back to my 10:00:11

17     earlier question, do you agree -- I'm going to ask 10:00:14

18     it again.  Do you agree or disagree that biopsy is 10:00:15

19     the ultimate gold standard which tells us -- tells 10:00:18

20     us that there is rejection going on or not?        10:00:20

21              MS. HABERNY:  Objection to form.           10:00:23

22              MR. DeJONG:  Objection to form, asked and  10:00:23

23     answered.                                          10:00:24

24              THE WITNESS:  I mean, in terms of the      10:00:26

25     kidney, it clearly is the most used gold standard, 10:00:27
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1	yes, for kidney. It always depends on the organ.	10:00:31
2	MR. WALTER: Q. Okay. What about other	10:00:34
3	organs?	10:00:35
4	A. When you get to organs like the pancreas	10:00:39
5	or the lung, then other technologies may be just as	10:00:43
6	important.	10:00:48
7	Q. For which organs is biopsy the ultimate	10:00:51
8	gold standard?	10:00:54
9	MS. HABERNY: Objection to form.	10:00:55
10	THE WITNESS: As I said, it's one of the	10:00:58
11	organs it's the ultimate gold standard. Always	10:00:59
12	depends on what you're looking for and what you want	10:01:03
13	to try to achieve.	10:01:05
14	MR. WALTER: Q. So biopsy's not the gold	10:01:12
15	standard for any organs?	10:01:14
16	MS. HABERNY: Objection to form.	10:01:16
17	THE WITNESS: As I said, it's the gold	10:01:17
18	standard for all the organs, but if this is the	10:01:18
19	ultimate gold standard, that's a different question.	10:01:21
20	MR. WALTER: Q. Okay. What is the	10:01:24
21	difference, in your mind, between the gold standard	10:01:25
22	and the ultimate gold standard?	10:01:28
23	A. Gold standard always depends on what	10:01:32
24	you're trying to achieve and what you want the --	10:01:34
25	what to use the assay for. That's what it is. If	10:01:35

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1	you want to see if an assay can predict or match	10:01:37
2	biopsy results, then yes, you have to use the biopsy	10:01:44
3	as a gold standard. If you're looking at a	10:01:48
4	functional -- let's say a injury -- functional	10:01:53
5	injury or impairment of organ injury due to	10:01:59
6	rejection, then you would check -- you would use a	10:02:02
7	different gold standard.	10:02:05
8	You know, it always depends on what you're	10:02:07
9	looking at. I mean, you have the example right	10:02:09
10	there in the claim. I mean, when you look at the	10:02:14
11	surveillance methods for cardiac allograft	10:02:21
12	vasculopathy, I mean, that's a form of rejection or	10:02:25
13	a presentation -- the clinical presentation to which	10:02:32
14	rejection contributes.	10:02:35
15	So in this case you would use a completely	10:02:36
16	different gold standard than if you compare to the	10:02:41
17	immunology in the cardiac tissue where of course you	10:02:47
18	would use a biopsy then. It always --	10:02:53
19	Q. What is the gold standard for transplant	10:02:58
20	rejection?	10:03:00
21	MS. HABERNY: Objection to form.	10:03:01
22	THE WITNESS: As I said, in many cases or	10:03:06
23	most the cases, you would use a biopsy, but it	10:03:08
24	really depends on the organ. For example, if you	10:03:12
25	have the pancreas, then biopsies are not all that	10:03:15

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  1    terribly important anymore.                        10:03:19

  2            MR. WALTER:  Q.  What is the gold standard  10:03:21

  3    for pancreas, if it's not biopsy?                  10:03:24

  4    A.        I mean, you look at the function.  You    10:03:29

  5    look at, for example, insulin production and these 10:03:30

  6    kinds of things.  I mean, that's what you're looking 10:03:33

  7    at.                                                 10:03:34

  8            You always have to be careful with          10:03:35

  9    biopsies.  I mean, biopsies are not the ultimate -- 10:03:37

 10    the ultimate diagnostic tool because a biopsy does  10:03:42

 11    not necessarily show you what's going on, because   10:03:45

 12    rejections are in many cases focal, and when you put 10:03:50

 13    a needle into an organ, if you get lucky, you hit   10:03:53

 14    the spot where the action is.  If you're not lucky, 10:03:57

 15    then you hit a spot where not much is going on.  And 10:04:00

 16    so a biopsy is also not 100% reliable.              10:04:06

 17    Q.        Would a person of ordinary skill in the   10:04:11

 18    art know what the gold standard is for transplant   10:04:13

 19    rejection?                                          10:04:15

 20            MR. DeJONG:  Objection to form.             10:04:17

 21            THE WITNESS:  A person of skill in the art  10:04:19

 22    would certainly know that, in terms of gold         10:04:21

 23    standards, it depends on what you want to test.  It 10:04:27

 24    depends on what you want to achieve, and the person 10:04:29

 25    of skill of art would know that there are several   10:04:32
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1	options for gold standards.	10:04:37
2	MR. WALTER: Q. And they would know what	10:04:38
3	those are?	10:04:39
4	MR. DeJONG: Objection; form.	10:04:41
5	THE WITNESS: The person of skill in the	10:04:43
6	art would clearly know that there is several	10:04:44
7	options, and the person of skill in the art would be	10:04:49
8	aware of what these options are, yes.	10:04:51
9	MR. WALTER: Q. Okay. And they would	10:04:53
10	know what they are on an organ-by-organ basis?	10:04:55
11	MS. HABERNY: Objection to form.	10:04:59
12	THE WITNESS: As I said before, there is	10:05:01
13	no such thing as a gold standard for a specific	10:05:03
14	organ. It always depends on what kind of test you	10:05:05
15	have, what is your index test, and depending on the	10:05:09
16	index test, you choose the gold standard, and there	10:05:11
17	may be several options for a gold standard.	10:05:16
18	MR. WALTER: Q. And the person of	10:05:20
19	ordinary skill in the art would know that; is that	10:05:21
20	right?	10:05:22
21	A. A person of skill in the art would know	10:05:25
22	that this is a complex question, yes.	10:05:26
23	Q. Okay. Well, let me try to ask the	10:05:29
24	question more specifically. Would the person of	10:05:31
25	ordinary skill in the art, for instance, in the	10:05:33

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1	context of a kidney transplant and trying to test	10:05:37
2	for transplant rejection of that kidney, would they	10:05:41
3	know what the gold standard would be?	10:05:44
4	MS. HABERNY: Objection to form.	10:05:47
5	THE WITNESS: They certainly would know	10:05:49
6	what the options are, yes.	10:05:50
7	MR. WALTER: Q. Okay. Same question for	10:05:52
8	pancreas?	10:05:58
9	A. Person of skill in the art would know what	10:05:59
10	the options are.	10:06:01
11	Q. And they would know that for both kidney	10:06:02
12	and pancreas, the most commonly used option is --	10:06:04
13	well, let me take kidney. For kidney, they would	10:06:07
14	know that the most commonly used option is biopsy,	10:06:10
15	right?	10:06:13
16	MS. HABERNY: Objection to form.	10:06:14
17	MR. DeJONG: Objection to form.	10:06:14
18	THE WITNESS: The most frequently chosen	10:06:15
19	option is biopsy, yes.	10:06:17
20	MR. WALTER: Q. And what is the most	10:06:20
21	frequently chosen option for pancreas?	10:06:21
22	MR. DeJONG: Objection to form.	10:06:24
23	MS. HABERNY: Objection to form.	10:06:24
24	THE WITNESS: I don't know.	10:06:27
25	MR. WALTER: Q. Would -- sorry. Go	10:06:29

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1	ahead.	10:06:31
2	A. I would have to look that up. The only	10:06:32
3	thing I can tell you is that the biopsy of a	10:06:33
4	pancreas is very dangerous, so I would have to ask	10:06:36
5	the experts how much they do biopsies and how	10:06:39
6	hesitant they are doing it.	10:06:43
7	Q. And what about the most commonly used	10:06:47
8	reference standard for heart transplant rejection?	10:06:53
9	Would the person of ordinary skill know what that	10:06:55
10	is?	10:06:58
11	MS. HABERNY: Objection to form.	10:07:00
12	MR. DeJONG: Objection to form.	10:07:01
13	THE WITNESS: That's more difficult to	10:07:02
14	answer. I mean, the person of skill in the art	10:07:04
15	definitely would know what the options are, and	10:07:08
16	there are several options.	10:07:12
17	MR. WALTER: Q. Okay. And what is the	10:07:13
18	most commonly used option; do you know?	10:07:14
19	MS. HABERNY: Objection to form.	10:07:16
20	THE WITNESS: I would think that this is	10:07:18
21	probably a tie between functional monitoring and --	10:07:22
22	so for example, using an angiography, coronary	10:07:28
23	angiography, would be one of the options.	10:07:35
24	Echocardiography would be an option. Biopsy may be	10:07:37
25	another option. So there are several options here.	10:07:40

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1	MR. WALTER: Q. Okay. And the person of	10:07:43
2	ordinary skill in the art would know those options?	10:07:44
3	MS. HABERNY: Objection to form.	10:07:47
4	THE WITNESS: Yes.	10:07:48
5	MR. WALTER: Q. Okay. And what about	10:07:50
6	liver? What is the most commonly used reference	10:07:53
7	standard for liver transplant rejection?	10:07:56
8	MR. DeJONG: Objection to form.	10:07:58
9	THE WITNESS: Most commonly or more	10:08:03
10	frequently -- most frequently used is the biopsy.	10:08:05
11	MR. WALTER: Q. And the person of	10:08:07
12	ordinary skill in the art would know that?	10:08:08
13	MS. HABERNY: Objection to form.	10:08:11
14	THE WITNESS: I said the person of art	10:08:15
15	would be aware of all the options for a potential	10:08:16
16	gold standard in the liver.	10:08:19
17	MR. WALTER: Q. And they would understand	10:08:21
18	that biopsy's the most commonly used standard?	10:08:22
19	MR. DeJONG: Objection to form.	10:08:28
20	THE WITNESS: It would be the most	10:08:34
21	frequently used standard, but there are other	10:08:35
22	standards.	10:08:37
23	MR. WALTER: Q. Okay. And what about	10:08:37
24	lung? What is the most frequently used standard for	10:08:45
25	lung transplant rejection?	10:08:48

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1	MS. HABERNY: Objection to form.	10:08:49
2	THE WITNESS: Gold standard for lung	10:08:58
3	transplant rejection. I don't know. I don't have	10:08:59
4	much experience with lung transplantation.	10:09:00
5	MR. WALTER: Q. Okay.	10:09:03
6	A. I would have to look it up.	10:09:04
7	MS. HABERNY: Dr. Christians, we've been	10:09:21
8	going for about an hour. Do you want to take a	10:09:22
9	break?	10:09:24
10	THE WITNESS: Break is always good.	10:09:28
11	MR. WALTER: All right. Take a ten-minute	10:09:30
12	break.	10:09:31
13	THE VIDEOGRAPHER: This is the end of	10:09:32
14	Media No. 1. Off the record at 11:09 a.m.	10:09:33
15	(Recess taken from 11:09 a.m. to	10:30:40
16	11:31 a.m.)	10:30:40
17	THE VIDEOGRAPHER: We are back on the	10:31:08
18	record at 11:31 a.m. This is the beginning of Media	10:31:08
19	No. 2.	10:31:12
20	MR. WALTER: Q. Dr. Christians, during	10:31:17
21	the break, did you discuss the substance of your	10:31:18
22	testimony with counsel?	10:31:21
23	A. I didn't discuss anything. I was gone	10:31:24
24	with the dog.	10:31:26
25	Q. All right. For kidney transplant	10:31:29

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1	transplant rejection, what reference standard would	10:38:49
2	you use?	10:38:51
3	MS. HABERNY: Objection to form.	10:38:53
4	MR. DeJONG: Objection to form.	10:38:53
5	THE WITNESS: That I have -- I have not	10:38:59
6	much experience with lung transplantation. I have	10:38:59
7	to ask my colleagues.	10:39:03
8	MR. WALTER: Q. For assessing the	10:39:04
9	sensitivity of an assay for pancreas transplant	10:39:05
10	rejection, what reference standard would you use?	10:39:11
11	MR. DeJONG: Objection; form.	10:39:13
12	THE WITNESS: Again, I don't know how	10:39:14
13	common it is to biopsy a pancreas because of the	10:39:17
14	risks, so it could be an imaging technology. Could	10:39:20
15	be just a functional assay to look at antibodies or	10:39:23
16	look at insulin or something like that. I don't	10:39:27
17	know. Could be different options for pancreas.	10:39:33
18	MR. WALTER: Q. Do you know -- do you	10:39:37
19	know what the reference standard would be for	10:39:38
20	assessing the sensitivity of an assay for pancreas	10:39:40
21	transplant rejection, or is that something you'd	10:39:46
22	have to go investigate with your colleagues?	10:39:47
23	MR. DeJONG: Objection to form.	10:39:50
24	THE WITNESS: As I said, I would have to	10:39:52
25	discuss that with my colleagues. They're -- I know	10:39:55

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1	that there are several options.	10:39:58
2	MR. WALTER: Q. For assessing the	10:40:00
3	sensitivity of an assay for detecting liver	10:40:02
4	transplant rejection, what reference standard would	10:40:04
5	you use?	10:40:07
6	MS. HABERNY: Objection to form.	10:40:08
7	THE WITNESS: Again, it really depends on	10:40:12
8	what you want to look at. I mean, if you want to	10:40:13
9	look at the bile ducts, which is -- of the targets	10:40:16
10	for transplantation, for rejection you would	10:40:19
11	probably use an imaging technology. If you want to	10:40:22
12	just look at general rejection, you would use a	10:40:25
13	biopsy. There could be different -- there are	10:40:28
14	different reference standards you may want to	10:40:31
15	consider depending on what the index assay is.	10:40:33
16	MR. WALTER: Q. When you say "index	10:40:36
17	assay," what do you mean?	10:40:38
18	A. Index assay is the assay you want to test.	10:40:39
19	Q. Okay. So the person of ordinary skill in	10:40:42
20	the art would understand that for the assay you're	10:40:43
21	trying to test, you would use a different reference	10:40:48
22	standard?	10:40:51
23	MR. DeJONG: Objection to form.	10:40:52
24	MS. HABERNY: Objection to form.	10:40:53
25	THE WITNESS: Yes. I mean, for every	10:40:54

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1	different assay, there may be a most appropriate	10:40:56
2	reference standard. It depends on which assay you	10:41:01
3	want to test.	10:41:04
4	MR. WALTER: Q. A person of ordinary	10:41:04
5	skill in the art would know that?	10:41:06
6	A. Yes.	10:41:10
7	MR. DeJONG: Objection to form.	10:41:10
8	MR. WALTER: Q. For detecting -- excuse	10:41:30
9	me. For assessing the sensitivity of an assay for	10:41:32
10	detecting graft dysfunction in organ transplant,	10:41:40
11	what reference standard would you use?	10:41:45
12	MS. HABERNY: Objection to form.	10:41:46
13	THE WITNESS: What graft dysfunction?	10:41:50
14	That's a functional assay. That really depends on	10:41:52
15	the organ. There's no general answer for this.	10:41:54
16	MR. WALTER: Q. Okay. Well, for kidney,	10:41:58
17	what would you use?	10:41:59
18	A. You have so many options. You could use	10:42:03
19	creatinine. You could use cystatin. You could use	10:42:06
20	one of the more recent protein kidney dysfunction	10:42:10
21	markers, such as KIM-1, NGAL, osteopontin.	10:42:36
22	Q. Okay. What is the most common reference	10:42:44
23	standard that's used for assessing the sensitivity	10:42:46
24	of an assay for kidney graft dysfunction in a kidney	10:42:53
25	transplant recipient?	10:42:59

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1	MS. HABERNY: Objection to form.	10:43:02
2	MR. DeJONG: Objection to form.	10:43:02
3	THE WITNESS: As I said, there is no	10:43:03
4	commonly accepted reference standard. I mean, most	10:43:05
5	of the assays are compared against GFR, but --	10:43:09
6	glomerular filtration rate, but everybody knows that	10:43:12
7	that is a very imperfect gold standard, so...	10:43:18
8	MR. WALTER: Q. For detecting -- excuse	10:43:25
9	me. For assessing the sensitivity of an assay for	10:43:29
10	graft dysfunction in a heart transplant recipient,	10:43:35
11	what is the reference standard?	10:43:39
12	MR. DeJONG: Objection to form.	10:43:41
13	THE WITNESS: Again, there is no single	10:43:44
14	reference standard. I mean, when you go and look at	10:43:45
15	the recommendations by the International Society of	10:43:53
16	Heart and Lung Transplantation, it could be a	10:43:58
17	coronary angiography in combination with	10:44:03
18	echocardiography. A lot of these recommendations	10:44:06
19	are combin -- are the combinations of different	10:44:08
20	assays.	10:44:13
21	So there's all kinds of echocardiographies	10:44:13
22	in terms of -- or angiographies. And then of course	10:44:16
23	you have the -- you have the -- when you want to	10:44:24
24	look at the function of the coronary arteries, there	10:44:28
25	is, as I mentioned, the intravascular ultrasound.	10:44:31

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1	So there are so many options in --	10:44:36
2	MR. WALTER: Q. And the person of	10:44:38
3	ordinary skill in the art would be aware of those	10:44:39
4	recommendations and options?	10:44:41
5	MR. DeJONG: Objection to form.	10:44:43
6	THE WITNESS: Yes, because there are	10:44:44
7	consensus documents, as I said, from the	10:44:46
8	International Society of Heart and Lung	10:44:48
9	Transplantation which discusses those.	10:44:50
10	MR. WALTER: Q. You said "consensus	10:44:52
11	documents"?	10:44:53
12	A. Yes.	10:44:54
13	Q. Okay. For detecting -- excuse me. For	10:44:55
14	assessing the sensitivity of an assay for detecting	10:45:04
15	graft dysfunction in a pancreas transplant	10:45:08
16	recipient, what reference standard would you use?	10:45:12
17	MR. DeJONG: Objection to form.	10:45:15
18	MS. HABERNY: Objection to form.	10:45:15
19	THE WITNESS: I said you have many options	10:45:19
20	there. I mean, you typically would monitor glucose	10:45:21
21	levels, insulin levels. As function for the -- of	10:45:25
22	the pancreas transplant, that would probably be the	10:45:32
23	easiest -- that's the easiest way of monitoring the	10:45:36
24	pancreas. Pancreas is tough to monitor.	10:45:37
25	MR. WALTER: Q. Person of ordinary skill	10:45:40

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1	in the art would know about those options?	10:45:41
2	A. Yes.	10:45:43
3	MS. HABERNY: Objection; form.	10:45:44
4	MR. WALTER: Q. Okay. For detecting --	10:45:45
5	excuse me. For assessing the sensitivity of an	10:45:52
6	assay for detecting graft dysfunction in a lung	10:45:55
7	transplant recipient, what reference standard would	10:45:59
8	you use?	10:46:01
9	MR. DeJONG: Objection to form.	10:46:03
10	THE WITNESS: Again, there is many	10:46:07
11	options. I mean, you could use imaging. You could	10:46:09
12	use functional assays, like looking at -- I mean,	10:46:14
13	there are so many ways of looking at lung function.	10:46:24
14	MR. DeJONG: Could we go off the record	10:46:33
15	just one moment, please?	10:46:35
16	THE VIDEOGRAPHER: This is the end of	10:46:36
17	Media No. 2. Off the record at 11:46 a.m.	10:46:37
18	(Interruption in the proceedings.)	10:49:08
19	THE VIDEOGRAPHER: We are back on the	10:49:17
20	record at 11:49 a.m. This is the beginning of Media	10:49:18
21	No. 3.	10:49:21
22	MR. WALTER: Q. All right. Before we	10:49:24
23	went off the record briefly, you identified the	10:49:25
24	options that could be used as a reference standard	10:49:28
25	for detecting -- for assessing the sensitivity of an	10:49:31

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1	assay for detecting graft dysfunction of a lung	10:49:36
2	transplant recipient, and my question for you is	10:49:39
3	would a person of ordinary skill in the art know	10:49:44
4	about those reference standards?	10:49:46
5	MS. HABERNY: Objection to form.	10:49:47
6	THE WITNESS: Yes, the person of skill in	10:49:50
7	the art would know about the options.	10:49:53
8	MR. WALTER: Q. Okay. For assessing the	10:49:56
9	sensitivity of an assay for detecting graft	10:50:00
10	dysfunction in a liver transplant recipient, what	10:50:04
11	reference standard would you use?	10:50:07
12	MR. DeJONG: Objection; form.	10:50:10
13	THE WITNESS: The function would -- I	10:50:14
14	mean, you would -- there is all kinds of things you	10:50:16
15	can use, like the liver enzymes. You could use	10:50:19
16	imaging technologies. You could use ultrasound. I	10:50:24
17	mean, there's all kind of things you could look at	10:50:29
18	in terms of liver dysfunction or structural changes	10:50:32
19	that impair liver function. For example, the	10:50:40
20	construction of the bile ducts and these kinds of	10:50:46
21	things.	10:50:48
22	MR. WALTER: Q. And the person of	10:50:48
23	ordinary skill in the art would know about those	10:50:49
24	reference standards?	10:50:51
25	MS. HABERNY: Objection to form.	10:50:52

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1	THE WITNESS: The person of skill of the	10:50:54
2	art would know about the options, yes.	10:50:55
3	MR. WALTER: Q. And what is the most	10:50:58
4	commonly used reference standard for assessing the	10:51:07
5	sensitivity of an assay for graft dysfunction in a	10:51:10
6	liver transplant recipient?	10:51:13
7	MR. DeJONG: Objection to form.	10:51:17
8	THE WITNESS: I -- my guess is that	10:51:21
9	bilirubin would definitely be one of the most	10:51:23
10	frequently used options, but there is many choices.	10:51:26
11	Always depends on what you want to look at, which	10:51:31
12	stage of dysfunction you want to look at or which	10:51:33
13	aspect of dysfunction you look at. I mean, the	10:51:38
14	liver does so many different things, and so there's	10:51:41
15	a whole plethora of different options to compare	10:51:45
16	with.	10:51:49
17	MR. WALTER: Q. Okay. And the person of	10:51:51
18	ordinary skill in the art would know how to vary the	10:51:52
19	reference standard depending on the particular thing	10:51:55
20	they're trying to look at?	10:51:57
21	MS. HABERNY: Objection to form.	10:51:58
22	MR. DeJONG: Objection to form.	10:51:59
23	THE WITNESS: The person of skill in the	10:52:03
24	art would know about the options.	10:52:05
25	MR. WALTER: Q. Would they know how to	10:52:06

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1	choose a different option depending on the	10:52:08
2	particular thing they were trying to look at?	10:52:10
3	MS. HABERNY: Objection to form.	10:52:13
4	MR. DeJONG: Objection to form.	10:52:13
5	THE WITNESS: Yeah, the person of skill in	10:52:16
6	the art would know about which of these options	10:52:17
7	would sort of, you know, be the narrow choice of	10:52:21
8	what would be appropriate and what would not be.	10:52:28
9	MR. WALTER: Q. And is that true for all	10:52:33
10	of the different types of organs we have been	10:52:34
11	discussing today?	10:52:38
12	MR. DeJONG: Objection to form.	10:52:40
13	THE WITNESS: Yes.	10:52:47
14	MR. WALTER: Q. All right. Earlier we	10:53:06
15	were talking about CAV. Is that different from	10:53:07
16	atherosclerosis?	10:53:16
17	A. Yes.	10:53:23
18	Q. Okay. How are they different?	10:53:23
19	A. I mean, atherosclerosis is something that	10:53:30
20	happens in any patient, independent of if they have	10:53:33
21	a heart transplant or not. CAV is restricted to	10:53:39
22	heart transplant patients.	10:53:43
23	Q. Are the symptoms of CAV in a heart	10:54:07
24	transplant patient different from the symptoms in	10:54:10
25	atherosclerosis?	10:54:14

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1	MR. DeJONG: Objection to form.	10:54:17
2	THE WITNESS: I mean, in the end, what it	10:54:19
3	always comes down to is ischemia, and that is the	10:54:21
4	same in a heart transplant patient as it is in a	10:54:27
5	lung transplant patient. But there are definitely	10:54:36
6	differences that are caused by the fact that this is	10:54:40
7	a heart transplant.	10:54:42
8	MR. WALTER: Q. If you were talking about	10:54:44
9	atherosclerotic coronary disease in a cardiac	10:54:46
10	transplant patient, would that be the same thing as	10:54:51
11	CAV?	10:54:53
12	MR. DeJONG: Objection to form, outside	10:54:54
13	the scope.	10:54:55
14	THE WITNESS: I mean, atherosclerosis is	10:55:02
15	one possible presentation of CAV.	10:55:06
16	MR. WALTER: Q. If a heart transplant	10:55:09
17	patient had atherosclerosis, would you say that they	10:55:12
18	had CAV?	10:55:16
19	MS. HABERNY: Objection to form, outside	10:55:17
20	the scope.	10:55:18
21	THE WITNESS: As I said, it's one form of	10:55:23
22	presentation. It's -- I mean, it's a vasculopathy,	10:55:25
23	and it's a kind of allograft that the patient has.	10:55:30
24	So I would guess that this would be part of CAV that	10:55:35
25	the patient has.	10:55:39

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1	wasn't out, but I mean, a consensus document is	11:04:15
2	always based on common practice, and since this	11:04:18
3	was -- was published only a couple month after the	11:04:22
4	priority date of the patent, I would guess that POSA	11:04:29
5	would have definitely considered this combination	11:04:35
6	here as one of the options.	11:04:39
7	Q. Okay. Take a look at the "Background"	11:04:41
8	section. It says "Angiography" is the first	11:04:43
9	section.	11:04:49
10	A. Right.	11:04:50
11	MR. DeJONG: Counsel, what page are you	11:04:52
12	looking at?	11:04:53
13	MR. WALTER: It's page A-1549.	11:04:55
14	Q. It states, "Coronary angiography has been	11:05:01
15	the cornerstone of the diagnosis of CAV vasculopathy	11:05:04
16	before the advent of IVUS." Do you see that?	11:05:07
17	A. Yes.	11:05:12
18	Q. Okay. Was that true in 2009?	11:05:13
19	A. Well, this is a historic background, so	11:05:18
20	what they explain and which was commonly known at	11:05:23
21	the time was that coronary -- the cardiac allograft	11:05:26
22	vasculopathy was actually found using coronary	11:05:33
23	angiography. So it was defined based on coronary	11:05:38
24	angiography, but then later, different technologies	11:05:43
25	were used to diagnosis.	11:05:46

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1	And this is what this whole document is	11:05:48
2	about, is that basically now there are more	11:05:50
3	technologies, and they try to define what the roles	11:05:55
4	of those technologies in the diagnosis of cardiac	11:05:57
5	allograft vasculopathy is.	11:06:03
6	Q. Okay. So let's take a look at that.	11:06:12
7	Let's take a look at page A-1551.	11:06:13
8	A. All right.	11:06:25
9	Q. There is a section entitled "IVUS	11:06:26
10	imaging," and I want you to look at the last	11:06:29
11	paragraph of that section that begins with the word	11:06:32
12	"Thus." Do you see that paragraph?	11:06:34
13	A. I do.	11:06:35
14	Q. Okay. It states, "Thus, although IVUS	11:06:41
15	remains an experimental tool to help investigators	11:06:47
16	evaluate the outcome of various therapeutic	11:06:49
17	conditions, clinical utility is limited, and	11:06:53
18	importantly, may be used at any point in the	11:06:57
19	transplant process for excluding significant disease	11:06:58
20	when the angiogram appears ambiguous." Do you see	11:07:02
21	that?	11:07:05
22	A. Yes, I do.	11:07:05
23	Q. Okay. Was it accurate in 2009 that IVUS	11:07:06
24	was an experimental tool?	11:07:10
25	MR. DeJONG: Objection to form.	11:07:13

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1	THE WITNESS: Yes, it was.	11:07:18
2	MR. WALTER: Q. Okay. And was it	11:07:19
3	accurate in 2009 that the clinical utility of IVUS	11:07:26
4	was limited?	11:07:29
5	MR. DeJONG: Objection to form.	11:07:33
6	THE WITNESS: I mean, if it's in terms of	11:07:37
7	cardiac allograft vasculopathy, then that is	11:07:41
8	probably true.	11:07:44
9	MR. WALTER: Q. And the person of	11:07:47
10	ordinary skill in the art would have known that in	11:07:47
11	2009?	11:07:49
12	A. The person of skill in the art would have	11:07:52
13	been aware of the limitations of IVUS, yes.	11:07:55
14	Q. And they would have been aware in 2009	11:07:58
15	that IVUS was an experimental tool?	11:08:01
16	MR. DeJONG: Objection; form.	11:08:04
17	MS. HABERNY: Objection; form.	11:08:05
18	THE WITNESS: They would have known about	11:08:07
19	the limitations, but as I show in my report, IVUS	11:08:08
20	was used as gold standard in several studies.	11:08:13
21	MR. WALTER: Q. But the consensus	11:08:19
22	document describes IVUS as an experimental tool,	11:08:21
23	does it not?	11:08:22
24	A. As I said, that's -- that's what's written	11:08:27
25	here, and IVUS, just an angiography, they all have	11:08:30

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1	their advantages and they have their drawbacks.	11:08:33
2	Q. And the person of ordinary skill in the	11:08:35
3	art would have known in 2009 that IVUS was an	11:08:37
4	experimental tool?	11:08:39
5	MR. DeJONG: Objection to form.	11:08:41
6	THE WITNESS: Yes, they would have known	11:08:43
7	that there were limitations to IVUS.	11:08:46
8	MR. WALTER: Q. It states at the end of	11:08:49
9	this paragraph, "Although IVUS remains very	11:08:51
10	sensitive to define CAV, we cannot advocate routine	11:08:59
11	IVUS at this time because its value as a surrogate	11:09:04
12	marker remains investigational." Do you see that?	11:09:06
13	A. Yes, I see that.	11:09:10
14	Q. Okay. It was true in 2009 that IVUS's	11:09:12
15	value as a surrogate marker was an investigation,	11:09:17
16	correct?	11:09:21
17	MR. DeJONG: Objection.	11:09:21
18	MS. HABERNY: Objection to form.	11:09:22
19	THE WITNESS: Said that IVUS was around	11:09:27
20	and it was used. And I mean, with all diagnostic	11:09:28
21	technologies, I mean, we're always trying to find	11:09:32
22	out what their real value is, and that's what	11:09:34
23	they're saying here, that there was no final verdict	11:09:37
24	yet if it was -- what its true value is for	11:09:40
25	diagnosing CAV.	11:09:42

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1	MS. HABERNY: Objection to form.	11:12:42
2	MR. DeJONG: Form.	11:12:43
3	THE WITNESS: As I said, it really depends	11:12:44
4	on the study and what patients they looked at and	11:12:46
5	which questions they asked. The literature that I'm	11:12:51
6	aware of was, as I said, anywhere between 30, 40%	11:12:55
7	to -- up to almost 90%.	11:13:00
8	MR. WALTER: Q. What was the sensitivity	11:13:02
9	of IVUS for determining CAV?	11:13:13
10	MS. HABERNY: Objection to form.	11:13:18
11	THE WITNESS: Again, the sensitivities in	11:13:21
12	the publications are also not very consistent. I	11:13:24
13	mean, again, this is discussed in this Min-U-Script	11:13:29
14	here, that this also depends on the technique of	11:13:34
15	IVUS, how -- what is the length of coronary artery	11:13:36
16	you're investigating, how many arteries you're	11:13:41
17	looking at. And depending on this, the sensitivity	11:13:44
18	of IVUS can also be somewhere between 30% to up to	11:13:46
19	90% that's mentioned in this article here.	11:13:51
20	MR. WALTER: Q. So the person of ordinary	11:13:53
21	skill in the art would understand that the	11:13:55
22	sensitivities of coronary angiography and IVUS would	11:13:59
23	vary depending on the factors that you've just	11:14:03
24	identified, right?	11:14:06
25	MS. HABERNY: Objection to form.	11:14:06

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1	THE WITNESS: Yes.	11:14:08
2	MR. WALTER: Q. Okay. Have you ever	11:14:10
3	heard the term "standard of care"?	11:14:15
4	MR. DeJONG: Objection to form.	11:14:18
5	THE WITNESS: Yes.	11:14:19
6	MR. WALTER: Q. Okay. What does that	11:14:20
7	mean?	11:14:21
8	A. It means that you're following the	11:14:22
9	protocol which could be your institutional protocol.	11:14:26
10	It could also be based on the consensus documents of	11:14:29
11	international/national societies. I mean, "standard	11:14:37
12	of care" is a very -- it's not a very well defined	11:14:40
13	term. Really depends on what you're basing it on.	11:14:46
14	Q. Okay. Let's take a look at Exhibit 2,	11:15:52
15	which is the '652 patent. And I want you to look at	11:15:58
16	column 6, lines 8 to 12. Do you have Exhibit 2?	11:16:04
17	A. Yeah, I have it. I just need to get to	11:16:12
18	column 6.	11:16:15
19	Q. Okay. It states, "Current surveillance	11:16:32
20	methods for CAV lack adequate sensitivity or require	11:16:35
21	invasive procedures and the most commonly applied	11:16:39
22	method, coronary angiography, lacks sensitivity."	11:16:43
23	Do you see that?	11:16:49
24	A. Yes, I do.	11:16:50
25	Q. Okay. You agree that in 2009, the most	11:16:51

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1	common method for surveilling CAV was coronary	11:16:55
2	angiography, right?	11:17:00
3	MS. HABERNY: Objection to form.	11:17:02
4	THE WITNESS: Yes, that was the most	11:17:07
5	frequently used method.	11:17:08
6	MR. WALTER: Q. Okay. And that was the	11:17:10
7	standard of care, correct?	11:17:12
8	A. Yes.	11:17:21
9	Q. Okay. Now, it states here that "coronary	11:17:21
10	angiography lacks sensitivity." Do you see that?	11:17:25
11	A. That's what's written here.	11:17:32
12	Q. Okay. Now, you don't understand that to	11:17:33
13	mean that the sensitivity was zero, right?	11:17:34
14	MS. HABERNY: Objection to form.	11:17:39
15	THE WITNESS: Well, I mean, if you just	11:17:42
16	take it by the way it's stated here, it could	11:17:44
17	include zero, but I guess something that is standard	11:17:48
18	of care would definitely be better than zero.	11:17:52
19	MR. WALTER: Q. So how would someone of	11:17:55
20	skill in the art understand this reference to	11:17:57
21	coronary angiography lacking sensitivity if they	11:18:00
22	wouldn't understand it to mean zero?	11:18:03
23	MS. HABERNY: Objection; form.	11:18:07
24	THE WITNESS: I mean, this is a blanket	11:18:11
25	statement that really doesn't say much. So in this	11:18:12

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 1    case, I would probably look at the Kobashigawa        11:18:15

 2    publication and see what Dr. Kobashigawa had to say   11:18:21

 3    about this.                                           11:18:26

 4            MR. WALTER:  Q.  Okay.  But you wouldn't      11:18:45

 5    understand this to be a statement that coronary       11:18:46

 6    angiography has a sensitivity of zero.  You wouldn't  11:18:48

 7    think that as someone of skill in the art, right?     11:18:52

 8    A.       I -- see, the sensitivity is a -- is based   11:18:58

 9    on distribution statistics, at least some degree.     11:19:06

10    So I mean, if you look at the individual patient,     11:19:11

11    what does "lack of sensitivity" mean?  It means that  11:19:16

12    there is false negatives.  So for an individual       11:19:19

13    patient, yes, it could be zero because the patient    11:19:21

14    has the disease and it's not caught.  I mean...       11:19:24

15    Q.       You think this might be referring to the     11:19:28

16    fact that it might not be caught in an individual     11:19:30

17    patient?                                              11:19:33

18    A.       Yeah.  I mean, that's what it basically      11:19:34

19    is.                                                   11:19:36

20    Q.       All right.  So it's basically saying that    11:19:37

21    angiography for surveilling CAV is not perfect,       11:19:43

22    right?                                                11:19:48

23    A.       That's what it says.  And it says you're     11:19:48

24    missing patients.  That's what it says.               11:19:50

25    Q.       What is the gold standard for assessing      11:20:06
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1	that's also stated here, that it was a technology	11:21:58
2	that is available, and that was known to be more	11:22:02
3	sensitive than coronary angiography. So it was just	11:22:05
4	something that made sense, is to compare these two	11:22:15
5	technologies with each other. And depending on --	11:22:20
6	you could define one as the gold standard or the	11:22:27
7	other.	11:22:29
8	MR. WALTER: Q. We talked about this	11:22:29
9	earlier. IVUS was the one where the consensus	11:22:31
10	document identified it as experimental in 2010,	11:22:33
11	correct?	11:22:37
12	MS. HABERNY: Objection to form.	11:22:41
13	THE WITNESS: Yes, but I mean, you have to	11:22:42
14	look at what does "experimental" mean, and you have	11:22:43
15	to take the whole document into consideration. What	11:22:45
16	they basically say is that it had its limitation and	11:22:50
17	that more studies were needed to fully understand	11:22:53
18	the value -- clinical value of IVUS, which does not	11:22:56
19	mean that it was not very sensitive technology which	11:23:01
20	was generally recognized as having an advantage --	11:23:05
21	potential advantage over angiography.	11:23:11
22	MR. WALTER: Q. Let's go to your	11:23:19
23	declaration again. You refer to something called a	11:23:59
24	STARD checklist?	11:24:20
25	A. Yes.	11:24:25

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1	Q. What is that?	11:24:26
2	MS. HABERNY: Counsel, where are you	11:24:28
3	looking?	11:24:29
4	MR. WALTER: It's in paragraph 77.	11:24:31
5	THE WITNESS: All right.	11:24:45
6	MR. WALTER: Q. Okay. What is the STARD	11:24:45
7	checklist?	11:24:47
8	A. STARD was an international consortium for	11:24:49
9	the standardization of how studies have to be	11:24:52
10	conducted and described that were assessing medical	11:25:01
11	diagnostic performance.	11:25:04
12	Q. And would the person of ordinary skill in	11:25:10
13	the art in 2009 have been aware of these guidelines?	11:25:12
14	A. Yes.	11:25:17
15	Q. And would they have known how to apply	11:25:20
16	them?	11:25:24
17	MR. DeJONG: Objection to form.	11:25:24
18	THE WITNESS: Yes.	11:25:28
19	MR. WALTER: Q. Let's take a look at	11:25:28
20	paragraph 62 of your declaration.	11:25:50
21	A. All right.	11:26:04
22	Q. You state, "Third, the claim does not make	11:26:09
23	clear whether the sensitivity of the claimed assay	11:26:11
24	and the comparator current surveillance method	11:26:15
25	should be based on the same reference standard	11:26:19

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1	(which, as I explain in more detail below, would be	11:26:21
2	nonsensical)." Do you see that?	11:26:25
3	A. Yes.	11:26:27
4	Q. Okay. So you refer to "the claimed assay"	11:26:27
5	there. What are you referring to when you say "the	11:26:29
6	claimed assay" in paragraph 62?	11:26:31
7	A. The claimed assay is the assay based on	11:26:34
8	donor-derived cell-free DNA.	11:26:39
9	Q. Okay. And then it says "the comparator	11:26:40
10	current surveillance method." What is that?	11:26:42
11	A. Well, this is the current surveillance	11:26:46
12	method for coronary -- for cardiac allograft	11:26:49
13	vasculopathy that is mentioned in claim 1(d).	11:26:54
14	Q. And you say that it would be nonsensical	11:26:56
15	to use the same reference standard for those two	11:26:59
16	assays, correct?	11:27:04
17	A. That is correct.	11:27:07
18	Q. Okay. Why would that be nonsensical?	11:27:07
19	A. Because the donor-derived cell-free DNA	11:27:12
20	assay is a biochemical assay which is linked or	11:27:16
21	driven by the pathobiochemistry of the rejection	11:27:22
22	process, while the cardio allograft vasculopathy	11:27:27
23	surveillance method would be a functional assay.	11:27:32
24	And so the reference standard for a functional assay	11:27:35
25	would be different than a reference standard for a	11:27:39

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 1    biochemical assay.                                  11:27:40

 2    Q.          So the person of ordinary skill in the art   11:27:46

 3    would know that it would be nonsensical to use the    11:27:48

 4    same reference standard for those two assays,        11:27:50

 5    correct?                                             11:27:55

 6              MR. DeJONG:  Objection to form.            11:27:56

 7              THE WITNESS:  It wouldn't have made much    11:28:01

 8    sense to the person of ordinary skill in the art,    11:28:03

 9    yes.                                                 11:28:05

10              MR. WALTER:  Q.  Now, is it your claim     11:28:05

11    that for a patent claim to avoid indefiniteness, the  11:28:16

12    specification needs to tell people not to adopt       11:28:22

13    interpretations that people of skill in the art       11:28:27

14    would find nonsensical?                               11:28:29

15              MS. HABERNY:  Objection to form.           11:28:31

16              MR. DeJONG:  Objection to form.            11:28:32

17              THE WITNESS:  That is a complicated        11:28:35

18    question.  Could you break this down for me, please?  11:28:35

19              MR. WALTER:  Q.  Sure.  Is it your         11:28:39

20    understanding that for patents to avoid              11:28:43

21    indefiniteness, the specifications need to tell      11:28:46

22    people not to adopt interpretations that people of   11:28:53

23    skill in the art would find nonsensical?            11:28:58

24              MS. HABERNY:  Objection to form.           11:29:01

25              MR. DeJONG:  Objection to form, calls for  11:29:02
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1	a legal conclusion.	11:29:03
2	THE WITNESS: Yeah, I mean, my	11:29:07
3	understanding is that the person of skill in the art	11:29:08
4	would need to know how to practice the claim. So --	11:29:12
5	and to -- of course, to practice something that is	11:29:14
6	nonsensical would not -- would not be feasible to	11:29:23
7	practice the claim for the person of skill in the	11:29:27
8	art. That's my understanding.	11:29:30
9	MR. WALTER: Q. So let me try -- I'll try	11:29:38
10	to break it down even further. The claims refer to	11:29:39
11	the dd-cfDNA assay, and they refer to the current	11:29:42
12	surveillance methods for CAV, correct?	11:29:48
13	A. That is correct.	11:29:53
14	Q. And you say in paragraph 62 it would be	11:29:54
15	nonsensical to adopt the same reference standard for	11:29:56
16	those two things, right?	11:29:59
17	A. Yes, that's what I'm saying.	11:30:02
18	Q. Okay. Do you think the patent needs to	11:30:04
19	explicitly say that it would be nonsensical to do	11:30:07
20	that to avoid indefiniteness?	11:30:13
21	MS. HABERNY: Objection; form.	11:30:16
22	THE WITNESS: I believe what the patent	11:30:20
23	needs to say is to exactly specify what the	11:30:22
24	reference standards for each of these two methods	11:30:26
25	is. That's the whole point I'm making in my	11:30:29

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1	spec- -- the patent claims would need to be limited	12:34:38
2	to those different rejection categories you	12:34:43
3	identified to be definite?	12:34:45
4	MS. HABERNY: Objection to form.	12:34:47
5	THE WITNESS: Well, to be definite, the	12:34:49
6	POSA would need reasonable guidance to conduct or	12:34:53
7	plan a study that would determine the sensitivity.	12:34:57
8	And for this to -- to do this, there would need to	12:35:02
9	be at least some critical detail, and it is	12:35:10
10	important to know what rejection this sensitivity	12:35:13
11	refers to.	12:35:17
12	MR. WALTER: Q. That's what I'm trying to	12:35:19
13	figure out. I'm trying to figure out what level of	12:35:20
14	detail. So I'm asking some specific questions that	12:35:22
15	have straightforward answers, "yes" or "no." Would	12:35:24
16	the patent need to include an experiment describing	12:35:26
17	how to determine sensitivity for each of early onset	12:35:30
18	rejection, acute rejection, or chronic rejection for	12:35:34
19	the claims to be definite?	12:35:36
20	MR. DeJONG: Objection to form. And he's	12:35:38
21	asked -- you've asked this several times. You know,	12:35:39
22	he's already answered this question.	12:35:41
23	MR. WALTER: Kevin, that's a bad	12:35:44
24	objection. He hasn't answered it. It's a	12:35:46
25	straightforward question. He hasn't answered it	12:35:48

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1	directly. You can instruct him not to answer. That	12:35:50
2	would be an improper instruction. We'd go to the	12:35:51
3	court. But I'm entitled to get a straightforward	12:35:54
4	answer to the question.	12:35:56
5	MR. DeJONG: You're not entitled to repeat	12:35:57
6	the same question over and over again. Okay?	12:35:58
7	MR. WALTER: Look, the record is what it	12:36:00
8	is. I'm pretty happy that I haven't gotten a direct	12:36:02
9	answer. So yeah, I'm entitled to ask it again and	12:36:04
10	come back to it. And if you think that's a direct	12:36:07
11	answer, you can do what you need to do, but I'm	12:36:09
12	going to keep asking until I get a direct answer.	12:36:11
13	Q. Go ahead and answer the question.	12:36:17
14	A. Well, as I said before, I mean, you don't	12:36:18
15	need every single detail, but you need at least the	12:36:22
16	key parameters. So I hope that answers your	12:36:24
17	question.	12:36:29
18	Q. It doesn't. Okay. "Yes" or "no." "Yes"	12:36:30
19	or "no." Would you need to have an experiment	12:36:32
20	describing how to determine sensitivity for each of	12:36:35
21	early onset rejection, acute rejection, and chronic	12:36:37
22	rejection?	12:36:42
23	MS. HABERNY: Mr. Walter, you're yelling	12:36:42
24	at the witness now. Please.	12:36:44
25	MR. WALTER: I'm not yelling at anybody.	12:36:46

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1	THE WITNESS: I mean, in my opinion, you	12:36:49
2	don't need example. A list of key parameters would	12:36:50
3	definitely be acceptable, but I would have to look	12:36:53
4	at it, what this looks like. I mean, it's a "what	12:36:57
5	if" question. I don't know. It's not in the	12:37:01
6	patent, and it hasn't been done.	12:37:04
7	MR. WALTER: Q. And you haven't thought	12:37:06
8	about it, right?	12:37:07
9	MS. HABERNY: Objection to form.	12:37:08
10	THE WITNESS: I could tell you exactly	12:37:09
11	which parameters would be needed at least, at a	12:37:10
12	minimum, to do a fair comparison, to determine the	12:37:13
13	sensitivity.	12:37:18
14	MR. WALTER: Q. Is it your understanding	12:37:20
15	that the patents can't claim a particular	12:37:35
16	sensitivity unless the patent includes clinical	12:37:37
17	study data?	12:37:42
18	MS. HABERNY: Objection to form.	12:37:45
19	MR. DeJONG: Objection to form, scope,	12:37:45
20	legal conclusion.	12:37:48
21	THE WITNESS: Well, I just told you that	12:37:50
22	clinical study data would basically be an example.	12:37:54
23	And I mean, it would be helpful to have an example,	12:37:57
24	but as I answered before, it's unnecessary just to	12:38:00
25	list what the key parameters would already be very	12:38:05

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1	helpful and would probably solve the problem, but	12:38:07
2	it's not there.	12:38:10
3	MR. WALTER: Q. Okay. So you agree that	12:38:34
4	it doesn't have to include clinical study data,	12:38:35
5	correct?	12:38:39
6	MS. HABERNY: Objection to form.	12:38:40
7	THE WITNESS: As I told you, there doesn't	12:38:47
8	need to be an example which then reports clinical	12:38:48
9	study data.	12:38:52
10	MR. WALTER: Q. And there also doesn't	12:38:53
11	need to be an experiment for each of the different	12:38:54
12	types of rejections for the claims to be definite,	12:38:56
13	right?	12:39:00
14	MS. HABERNY: Objection to form.	12:39:00
15	MR. DeJONG: Objection to form.	12:39:01
16	THE WITNESS: I mentioned before, it would	12:39:08
17	be helpful to have such an experiment, but again, a	12:39:09
18	list of key parameters at a minimum would help here.	12:39:12
19	MR. WALTER: Q. Okay. But you don't need	12:39:17
20	to have an experiment for each of the different	12:39:18
21	types of rejections, right? The claims would still	12:39:20
22	be -- could still be definite without such a set of	12:39:22
23	experiments, right?	12:39:24
24	MS. HABERNY: Objection to form.	12:39:25
25	MR. DeJONG: Objection to form.	12:39:26

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1	THE WITNESS: It wouldn't need --	12:39:35
2	that's -- I mean, as long as it's sufficiently	12:39:37
3	described to allow to conduct the experiments in a	12:39:40
4	standardized way to know exactly what is claimed, I	12:39:44
5	think that's sufficient.	12:39:49
6	MR. WALTER: Q. Okay. And the kinds of	12:40:04
7	parameters that you think would be required would be	12:40:05
8	the kind of things that are in, for instance, the	12:40:07
9	STARD checklist?	12:40:10
10	A. The STARD checklist is definitely	12:40:14
11	something that would provide guidance how to do	12:40:18
12	this. I mean, the STARD checklist goes beyond that.	12:40:21
13	It's also about how to publish it and these kinds of	12:40:25
14	things. But most of the key param- -- or all of the	12:40:28
15	key parameters are in the STARD list.	12:40:31
16	Q. Let's go back to the patent.	12:41:05
17	A. All right.	12:41:10
18	Q. Let's take a look at column 16, lines 63	12:41:40
19	to 65. It reads --	12:41:45
20	A. I'm not there yet. Sorry.	12:41:51
21	Q. Okay.	12:41:53
22	A. I am not that fast here. 16. It was 63,	12:41:56
23	right?	12:42:14
24	Q. Yes.	12:42:15
25	A. All right. Got it. Okay.	12:42:16

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1	Objection to scope. This deposition is supposed to	13:09:01
2	be about his opinion on indefiniteness, not Section	13:09:04
3	101.	13:09:06
4	MR. WALTER: I think this is def- --	13:09:07
5	MS. HABERNY: Far outside the scope. No,	13:09:09
6	you're trying to get admissions on Section 101 here.	13:09:10
7	MR. WALTER: I'm not.	13:09:13
8	THE WITNESS: And what I really have a	13:09:21
9	problem with is the word "conventional" because my	13:09:23
10	suspicion here is that this is some sort of legal	13:09:26
11	term, which I'm not fully aware of what it truly	13:09:29
12	means. But I mean, if I just look here at the 56%,	13:09:32
13	and that an assay has a sensitivity of greater than	13:09:35
14	56%, and we ignore anything else here, everything	13:09:41
15	else here, I would say, you know, that's the bare	13:09:44
16	minimum of what a clinical assay that is usable	13:09:48
17	should have.	13:09:52
18	And at least the way I understand the word	13:09:53
19	"conventional," in a very normal way, I would say	13:09:56
20	yeah, I mean, the assay should have 56%. Otherwise,	13:10:00
21	it's useless. Is that the answer you wanted?	13:10:03
22	MR. WALTER: Q. Yeah.	13:10:10
23	A. Okay. Yeah, so I mean, with all the	13:10:11
24	limitations I just told you, the caveats, I would	13:10:17
25	say yeah, it's conventional. I mean, an assay	13:10:19

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1	should have 56% sensitivity.	13:10:24
2	MR. WALTER: All right. Let's take a	13:10:39
3	short break. I might be close to wrapping up.	13:10:40
4	THE VIDEOGRAPHER: This is the end of	13:10:42
5	Media No. 4. Off the record at 2:10 p.m.	13:10:43
6	(Recess taken from 2:10 p.m. to 2:34 p.m.)	13:28:35
7	THE VIDEOGRAPHER: We are back on the	13:33:52
8	record at 2:34 p.m. This is the beginning of Media	13:33:54
9	No. 5.	13:33:58
10	MR. WALTER: Q. Dr. Christians, is it	13:34:03
11	possible to say that a diagnostic test for a	13:34:07
12	particular condition can be more sensitive for the	13:34:10
13	purpose of detecting that condition than the	13:34:12
14	sensitivity of a diagnostic test for detecting	13:34:15
15	another condition?	13:34:19
16	MR. DeJONG: Objection to form.	13:34:21
17	THE WITNESS: I mean, you can compare the	13:34:31
18	numbers of sensitivities, but the question is does	13:34:34
19	it make sense.	13:34:39
20	MR. WALTER: Q. So you could have, for	13:34:41
21	instance, a pregnancy test that's 99% sensitive and	13:34:43
22	you could have a pneumonia test that's 85%	13:34:50
23	sensitive, and you could say that the pregnancy test	13:34:53
24	is more sensitive for detecting pregnancy than the	13:34:57
25	pneumonia test is for detecting pneumonia, right?	13:35:00

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1	MS. HABERNY: Objection to form.	13:35:03
2	MR. DeJONG: Objection to form.	13:35:04
3	THE WITNESS: Depends on what the context	13:35:09
4	of that comparison is. Wouldn't make any sense to	13:35:10
5	me to do that.	13:35:13
6	MR. WALTER: Q. But you could do it. You	13:35:15
7	could say that the 99% sensitivity is greater than	13:35:16
8	the 85% sensitivity, correct?	13:35:18
9	MR. DeJONG: Objection to form.	13:35:20
10	THE WITNESS: You can compare any number	13:35:23
11	you want to. A matter if it makes sense to do it.	13:35:24
12	MR. WALTER: Q. All right. I don't have	13:35:38
13	anything else.	13:35:39
14	MR. DeJONG: All right. We're going to	13:35:40
15	have to take a short break, and then we'll come back	13:35:41
16	and we'll let you know if we have any redirect.	13:35:44
17	THE VIDEOGRAPHER: This is the end of	13:35:48
18	Media No. 5. Off the record at 2:35 p.m.	13:35:49
19	(Recess taken from 2:35 p.m to 2:48 p.m.)	13:48:22
20	THE VIDEOGRAPHER: We are back on the	13:48:51
21	record at 2:48 p.m. This is the beginning of Media	13:48:52
22	No. 6.	13:48:56
23	MR. DeJONG: And no questions from	13:48:58
24	Defendant Eurofins.	13:49:00
25	MS. HABERNY: Yeah, no questions from	13:49:01

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1	Natera.	13:49:03
2	MR. DeJONG: So the deposition is closed.	13:49:06
3	THE VIDEOGRAPHER: This concludes today's	13:49:07
4	testimony given by Uwe Christians. Six media were	13:49:09
5	recorded and will be retained by Veritext Legal	13:49:14
6	Solutions. We are off the record at 2:49 p.m.	13:49:17
7	MR. WALTER: We'll need an expedited	13:49:28
8	transcript, just as quickly as you can get it.	13:49:30
9	MS. HABERNY: Natera will take the same.	13:49:32
10	MR. WALTER: And you know, if you can get	13:49:34
11	a rough, a rough would be good too.	13:49:34
12	THE REPORTER: Mr. DeJong, do you want a	13:49:34
13	separate copy?	13:49:34
14	MR. DeJONG: Yeah, I guess that's fine.	13:49:58
15	We don't need the expedited, but we would like to	13:50:01
16	get the rough. If we can get the expedited this	13:50:04
17	week, that's fine.	13:50:08
18	(Whereupon the deposition concluded at	
19	1:50 p.m.)	
20		
21	---oOo---	
22		
23		
24		
25		

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A0200

```
 1                    CERTIFICATE OF REPORTER

 2

 3            I, Natalie Y. Botelho, a Certified

 4    Shorthand Reporter, hereby certify that the witness

 5    in the foregoing deposition was by me duly sworn to

 6    tell the truth, the whole truth, and nothing but the

 7    truth in the within-entitled.

 8            The said deposition was taken down in

 9    shorthand by me, a disinterested person, at the time

10    and place therein stated, and that the testimony of

11    said witness was thereafter reduced to typewriting,

12    by computer, under my direction and supervision;

13            That before completion of the deposition,

14    review of the transcript [ ] was [X] was not

15    requested.  If requested, any changes made by the

16    deponent (and provided to the reporter) during the

17    period allowed are appended hereto.

18            I further certify that I am not of counsel

19    or attorney for either or any of the parties to the

20    said deposition, nor in any way interested in the

21    event of this cause, and that I am not related to

22    any of the parties thereto.

23    DATED: March 29, 2021

24    _____

25    Natalie Y. Botelho, CSR No. 9897
```
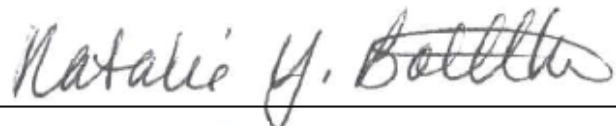
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Amendments to the Claims:

1-35.   (Cancelled)

36.   (Previously Presented) A method comprising:

(a)   providing a sample from a subject who has received a transplant from a donor;

(b)   conducting a multiplexed reaction on the sample to detect one or more nucleic acids derived from the transplant from the donor; and

(c)   diagnosing, predicting, or monitoring transplant status or outcome based on the detection of the one or more nucleic acids.

37.   (Cancelled)

38.   (Cancelled)

39.   (Currently Amended) The method of claim 36, wherein the one or more nucleic acids derived from the donor are detected based on a marker profile comprising one or more genetic variations selected from single nucleotide polymorphisms (SNPs), one or more variable number of tandem repeats (VNTRs), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, or one or more insertion elements; and

(a)   ~~diagnosing, predicting, or monitoring transplant status or outcome based on the detection of the one or more nucleic acids.~~

40.   (Currently Amended) The method of ~~any of~~ claims 36-~~37~~, wherein the one or more nucleic acids are cell-free nucleic acids.

41.   (Currently Amended) The method of ~~any of~~ claims 36-~~37~~, wherein the one or more nucleic acids is DNA.

42.   (Currently Amended) The method of ~~any of~~ claims 36-~~37~~, wherein detecting one or more nucleic acids derived from the transplant from the donor comprises quantifying the one or more nucleic acids.

43.   (Previously Presented) The method of claim 36, wherein the detecting one or more nucleic acids derived from the transplant from the donor comprises sequencing.

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44.   (Previously Presented) The method of claim 36, wherein the detecting one or more nucleic acids derived from the transplant from the donor comprises shotgun sequencing

45.   (Previously Presented) The method of claim 36, wherein the multiplexed reaction occurs in a single container.

46.   (Previously Presented) The method of claim 36, wherein the reaction to detect one or more nucleic acids comprises detecting at least ten different nucleic acids.

47.   (Currently Amended) The method of any one of claims 36, 37, or 38, further comprising administering an immunosuppressive drug.

48.   (Currently Amended) The method of claim 36 or 37, wherein diagnosing, predicting, or monitoring transplant status or outcome comprises treating a transplant rejection in a subject in need thereof.

49.   (Currently Amended) The method of claim 36 or 37, wherein diagnosing, predicting, or monitoring transplant status or outcome comprises determining, modifying, or maintaining an immunosuppressive regimen.

50.   (Currently Amended) The method of claim 36 or 37, wherein detecting one or more nucleic acids comprises detecting genetic variations.

51.   (Currently Amended) The method of claim 36 or 37, wherein detecting one or more nucleic acids derived from the transplant from the donor comprises conducting an assay selected from: digital PCR, real-time PCR, array, or any combination thereof.

52.   (Cancelled)

53.   (Previously Presented) The method of claim 50, wherein the genetic variations are selected from single nucleotide polymorphisms (SNPs), one or more variable number of tandem repeats (VNTRs), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, one or more insertion elements, or any combination thereof.

54.   (Previously Presented) The method of claim 36, wherein the one or more nucleic acids comprise at least one single nucleotide polymorphism.

55.   (Cancelled)

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56.   (Currently Amended) The method of ~~any one of~~ claims 36-~~38~~, wherein the sample is blood or serum.

57.   (Currently Amended) The method of claim 36 ~~or 37~~, wherein the sample is urine or stool.

58.   (Currently Amended)The method of claim 36 ~~or 37~~, <mark>wherein the sensitivity of the method is greater than 56%.</mark>

59.-65. (Cancelled)

66.   (Currently Amended) The method of 36 ~~or 59~~, wherein the transplant is selected from the group consisting of: kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

67.   (Currently Amended) The method of claim 36 ~~59 or 61~~, wherein the transplant is a heart transplant or kidney transplant.

68.   (Cancelled)

69.   (Cancelled)

A0204

UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/508,318	07/19/2012	Stephen R. Quake	STAN-706	6003

77974 7590 05/10/2013		

Stanford University Office of Technology Licensing
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East Palo Alto, CA 94303

EXAMINER
BUNKER, AMY M

ART UNIT	PAPER NUMBER
1639	

NOTIFICATION DATE	DELIVERY MODE
05/10/2013	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docket@bozpat.com
zuehlke@bozpat.com
zizzo@bozpat.com

PTOL-90A (Rev. 04/07)

A0205

	Application No.	Applicant(s)
Office Action Summary	13/508,318	QUAKE ET AL.
	Examiner	Art Unit	AIA (First Inventor to File) Status
	AMY M. BUNKER	1639	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on <u>21 March 2013</u>.
　☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
2a) ☐ This action is **FINAL**.　　2b) ☒ This action is non-final.
3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5) ☒ Claim(s) <u>36,39-51,53,54,56-58,66 and 67</u> is/are pending in the application.
　5a) Of the above claim(s) _____ is/are withdrawn from consideration.
6) ☐ Claim(s) _____ is/are allowed.
7) ☒ Claim(s) <u>36,39-51,53,54,56-58,66 and 67</u> is/are rejected.
8) ☐ Claim(s) _____ is/are objected to.
9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.
* If any claims have been determined <u>allowable</u>, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.
11) ☒ The drawing(s) filed on <u>04 May 2012</u> is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
　Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
　Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
　a) ☐ All　b) ☐ Some *　c) ☐ None of the:
　　1. ☐ Certified copies of the priority documents have been received.
　　2. ☐ Certified copies of the priority documents have been received in Application No. _____.
　　3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
　* See the attached detailed Office action for a list of the certified copies not received.
Interim copies:
　a) ☐ All　b) ☐ Some　c) ☐ None of the: Interim copies of the priority documents have been received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date <u>10/19/2012</u>.
3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
4) ☐ Other: _____.

U.S. Patent and Trademark Office
PTOL-326 (Rev. 03-13)　　　　　Office Action Summary　　　　　Part of Paper No./Mail Date 20130429

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Application/Control Number: 13/508,318   Page 2
Art Unit: 1639

DETAILED ACTION

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are currently pending. Claims 1-35 were previously canceled by Applicant's amendment filed 9-21-2012. Claims 36-69 were previously added by Applicant's amendment filed 9-21-2012. Claims 37, 38, 52, 55, 59-65, 68 and 69 were canceled by Applicant's amendment filed 3-21-2013.   Claims 39-42, 47-51, 56-58, 66 and 67 were amended by Applicant's amendment filed 3-21-2013.

Response to Election/Restriction

Applicant's election of Group I, claims 36, 39-51, 53, 54, 56-58, 66 and 67, with traverse and the election of species: (A) single nucleotide polymorphisms (B) kidney transplant in the reply filed on March 21, 2013 is acknowledged. The traversal is on the ground(s) that it would not be unduly burdensome to perform a search on all of the claims together in the present application (Applicant Remarks, pg. 6, second full paragraph). This is not found persuasive because search burden is not a criterion for restriction under 35 U.S.C. 121 and 372. Therefore, the requirement for species restriction is maintained.

No claims are withdrawn from consideration. Applicant timely traversed the restriction (election) requirement in the reply filed on March 21, 2013. Applicant timely responded to the election requirement in the Paper filed March 21, 2013.

The requirement is still deemed proper and is therefore made FINAL.

It is noted that when a final requirement for restriction is made by the examiner, applicant may file a petition under 37 CFR 1.144 for review of the restriction requirement. The propriety of a requirement to restrict, if traversed, is reviewable by petition under 37 CFR 1.144. *In re* Hengehold, 440 F.2d 1395, 169 USPQ 473 (CCPA 1971).

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are under consideration to which the following grounds of rejection are applicable.

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Application/Control Number: 13/508,318   Page 3
Art Unit: 1639

Priority

The present application is a 35 U.S.C. 371 national stage filing of International Application No. PCT/US2010/055604, filed on November 5, 2010, which claims the benefit of US Patent Application No. 61/280,674, filed on November 6, 2009.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on October 19, 2012 is not in compliance with the provisions of 37 C.F.R. § 1.98(a)(2), which requires a legible copy of each U.S. and foreign patent; each publication or that portion which caused it to be listed; and all other information or that portion which caused it to be listed.

The following reference has not been considered by the examiner, as indicated on Form PTO 1449.

a) Reference 121: Mandel *et al.*, of IDS filed on October 19, 2012 has not been considered because and English translation of the document has not been provided.

All other documents in said Information Disclosure Statement were considered as noted by the Examiner initials in the copy attached hereto.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

(B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

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Claims 36, 42-44 and 51 are vague and indefinite because they recite the phrase "derived from " and the metes and bounds of how one or more nucleic acids can be "derived from" the claimed transplant and still meet the intended limitation of the claims are not clear. Without a clear statement of the process by which the starting material is derivatized, it is not possible to know the metes and bounds of a "derivative" because any given starting material can have many divergent derivatives depending on the process of derivatization, especially since the claimed starting material encompasses tissues as diverse as kidney, lung , intestine, for example. This rejection could be overcome by substituting "isolated" for "derived" in the claim.

For the purpose of a compact prosecution the term "derived" recited in claims 36 and 42-44 has been interpreted as "obtained or isolated from."

Claims 36 (c) is indefinite in reciting "monitoring transplant status" in line 6. There is no antecedent basis for "transplant status" and it is unclear to what transplant the status corresponds, the transplanted organ, the subject who has received the transplant, specific samples from the subject who has received the transplant. Therefore, the metes and bounds of "monitoring transplant status" are indefinite.

Claims 39-41, 45-50, 53, 54, 56-58, 66 and 67 are indefinite insofar as they depend from claim 36.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary

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        skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

        This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

        Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are rejected under 35 U.S.C. 103(a) as being unpatentable over Lo Yuk-Ming *et al.* (U.S. Patent Application No. 20050282185, published December 22, 2005) in view of Saint-Mezard *et al.* (International Patent Application No. WO2009060035A1, published May 14, 2009).

        Lo Yuk-Ming *et al.* teach a method of differentiating DNA of an organ **donor** from **DNA** of an organ **recipient** using a **biological sample** such as plasma or serum (e.g., cell-free DNA) (instant claims 40, 41 and 56) in order to **predict** the clinical progress of the transplantation recipient especially applied to **organ rejection** (instant claim 36, in part) (paragraph [0030]), where DNA includes any sequence of more than one nucleotide such as polynucleotides, gene fragments and complete gene sequences, as well as, the study of **single nucleotide polymorphisms** (SNPs) (elected species), as well as, methylated and unmethylated alleles (e.g., genetic variations) (instant claims 50, 53 and 54) (paragraphs [0012], lines 6-8, [0019] and Fig. 2). Additionally, Lo Yuk-Ming *et al.* teach the detection of fetal DNA in a in a biological sample obtained from a mother and differentiating fetal DNA from maternal DNA based upon epigenetic markers such as DNA methylation (paragraph [0007], lines 4-7). Lo Yuk-Ming *et al.* teach that the assay is applicable to the study of cellular chimerism following **solid organ** transplantation (e.g., liver, spleen, heart, pancreas, and kidneys), post-translational plasma DNA chimerism and urinary DNA chimerism (instant claims 66 and 67) (paragraph [0044], lines 1-10). Lo Yuk-Ming *et al.* also teach that the method is based on the detection of differentially

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methylated DNA sequences detected by polymerase chain reaction (PCR) (paragraph [0027], lines 3-6). As an example, Lo Yuk-Ming *et al.* further teach the detection of post-bone marrow transplantation chimerism by isolating DNA from transplantation recipients and conducting methylation-specific PCR (paragraphs [0032] to [0037] and [0040] to [0041]), where the assay could be developed into a quantitative format using **real-time PCR** (instant claims 42 and 51), which would allow for the **monitoring** of levels of chimerism in a particular person and, in the case of **urinary** or **plasma** DNA chimerism, such an assay could be used for **monitoring graft rejection** (instant claim 57) (paragraph [0045]). Lo Yuk-Ming *et al.* also teach that fetal DNA in maternal plasma was detected using direct sequencing (instant claim 43) (paragraph [0014], lines 1-5 and Fig. 4); and that DNA sequencing on purified PCR products were analyzed using an ABI Prism 310 Genetic Analyzer (e.g., shotgun sequencing) (instant claim 44) (paragraph [0060]).

Lo Yuk-Ming *et al.* do not teach conducting a multiplexed reaction (instant claim 36, in part); or where the multiplexed reaction occurs in a single container (instant claim 45); or where the reaction to detect one or more nucleic acids comprises detecting at least ten different nucleic acids (instant claim 46); or where the method further comprises administering an immunosuppressive drug (instant claim 47); or where transplant status or outcome comprises treating a transplant rejection in a subject in need thereof (instant claim 48); or where the transplant status or outcome comprises modifying or maintaining an immunosuppressive regimen (instant claim 49); or where the sensitivity of the method is greater than 56% (instant claim 58).

Saint-Mezard *et al.* teach the analysis of genes to provide a molecular signature to accurately detect and/or grade transplant chronic rejection for the early **diagnosis** of allograft rejection (e.g., renal allograft rejection) (elected species) and new **prognostic** markers to minimize and personalize immunosuppression (paragraph [10], lines 1-5), as well as, methods for **monitoring** the status of a transplanted organ (paragraph [12], lines 1-2). Saint-Mezard *et al.* also teach a method of monitoring transplant rejection in a subject by detecting a level of gene expression (paragraph [21]), where the increased expression of at least 5, 10, 15 or 18 gene markers of any of the genes in Table 1 indicates transplant rejection (paragraph [13], lines 3-5, Table 1). Saint-Mezard *et al.* also teach the use of probe sets are a group of nucleic acids that may be used to detect two or more genes through amplification by PCR or RT-PCR such that a

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probe set may be in solution as would be typical for **multiplex PCR** or adhered to a solid surface as in an array or microarray (instant claim 36, in part) (paragraph [49], lines 1-3 and 9-10). Saint-Mezard *et al.* further teach prophylactic and therapeutic methods for preventing transplant rejection (paragraph [127], lines 1-2) associated with increased biomarker expression or activity by administering to the subject a compound or agent having **immunosuppressive** properties (instant claims 47 and 48) (paragraph [128]) such that information generated from more than one pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment of a subject (instant claim 49) (paragraph 137], lines 1-3).

The combined references of Lo Yuk-Ming *et al.* and Saint-Mezard *et al.* do not teach where the multiplexed reaction occurs in a single container (instant claim 45) or where the sensitivity of the method is greater than 56% (instant claim 58).

In view of the teachings of Lo Yuk-Ming *et al.*, which exemplify method of differentiating DNA of an organ donor from DNA of an organ recipient using a biological sample such as plasma or serum in order to predict the clinical progress of the transplantation recipient especially applied to organ rejection; and in view of the teachings of Saint-Mezard *et al.*, directed to prognostic markers to minimize and personalize immunosuppression (paragraph [10], lines 1-5), as well as, methods for monitoring the status of a transplanted organ, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to modify the method of monitoring bone marrow transplants as taught by Lo Yuk-Ming *et al.* to include the biomarkers as taught by Saint-Mezard *et al.* to monitor transplant rejection, select an immunosuppressive regimen, or determine the effectiveness of a treatment in a subject following transplant. Moreover, the combined references do not teach where the multiplexed reaction occurs in a single container; or where the sensitivity of the method is greater than 56%, however, one of ordinary skill in the art at the time the invention was made would understand that multiplexed reactions are often conducted on a single PCR mixture. In addition, Saint-Mezard *et al.* do teach that molecular diagnostics are more sensitive than current monitoring and diagnostic modalities and can be used as gene- or protein-based tests for the early or pre-clinical diagnosis of rejection (paragraph [8], lines 3-9); additionally, it is noted that *In re* Best (195 USPQ 430) and *In re* Fitzgerald (205 USPQ 594) discuss the support of rejections, wherein the

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prior art discloses subject matter which there is reason to believe naturally includes functions that are newly cited or is identical to a product instantly claimed. In such a situation, the burden is shifted to the Applicants to "prove that subject matter shown to be in the prior art does not possess characteristic relied on" (205 USPQ 594, second column, first full paragraph). Thus, the prior art teaches all of the limitations of the claimed invention.

Therefore, the invention, as a whole, was *prima facie* obvious to one of ordinary skill in the art.

Conclusion

Claims 36, 39-51, 53, 54, 56-58, 66 and 67 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to AMY M. BUNKER whose telephone number is 313-446-4833. The examiner can normally be reached on 7:00am - 4:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on 313-446-4833. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

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Examiner, Art Unit 1639

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/Maria Leavitt/

Primary Examiner, Art Unit 1633

Original Clinical Science—General

OPEN

Analytical Validation of a Single-nucleotide Polymorphism-based Donor-derived Cell-free DNA Assay for Detecting Rejection in Kidney Transplant Patients

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Background. Early detection of rejection in kidney transplant recipients holds the promise to improve clinical outcomes. Development and implementation of more accurate, noninvasive methods to detect allograft rejection remain an ongoing challenge. The limitations of existing allograft surveillance methods present an opportunity for donor-derived cell-free DNA (dd-cfDNA), which can accurately and rapidly differentiate patients with allograft rejection from patients with stable organ function. **Methods.** This study evaluated the analytical performance of a massively multiplexed polymerase chain reaction assay that targets 13 962 single-nucleotide polymorphisms, characterized and validated using 66 unique samples with 1064 replicates, including cell line-derived reference samples, plasma-derived mixtures, and transplant patient samples. The dd-cfDNA fraction was quantified in both related and unrelated donor-recipient pairs. **Results.** The dd-cfDNA assay showed a limit of blank of 0.11%, a limit of detection and limit of quantitation of 0.15% for unrelated donors, and limit of blank of 0.23%, a limit of detection and limit of quantitation of 0.29% for related donors. All other metrics (linearity, accuracy, and precision) were observed to be equivalent between unrelated and related donors. The measurement precision of coefficient of variation was 1.8% (repeatability, 0.6% dd-cfDNA) and was <5% for all the different reproducibility measures. **Conclusions.** This study validates the performance of a single-nucleotide polymorphism-based massively multiplexed polymerase chain reaction assay to detect the dd-cfDNA fraction with improved precision over currently available tests, regardless of donor-recipient relationships.

(*Transplantation* 2019;103: 2657–2665)

INTRODUCTION

Kidney transplantation is the best option for patients with end-stage renal disease.[1] According to the United Network for Organ Sharing, more than 19 000 kidneys were transplanted in the United States in 2016 and approximately 200 000 patients are living with a functional kidney transplant (KT).[1] Despite lifelong immunosuppressive maintenance regimens designed to optimize the therapeutic outcome,[2] approximately 20%–30% of patients experience overall renal graft failure within the first 5 years,[3] and only 55% of transplanted kidneys survive for 10 years.[4,5] Thus, a compelling need exists for new strategies to avoid or minimize acute or subclinical rejection episodes, nephrotoxicity, other comorbidities, and otherwise

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improve clinical outcomes.[6] Optimal implementation of new methods would require a simple, accurate way to monitor allograft health, allowing early detection of treatable pathology and with the goal of preventing graft loss by optimizing immunosuppressive regimens. Current clinical options to monitor allograft rejection in transplant recipients, most notably biopsies and assessing dynamic changes in serum creatinine (SCr), have significant drawbacks.

Biopsy with detailed pathology is the "gold standard" for the diagnosis of active rejection (AR). Although some centers recommend asymptomatic surveillance "protocol" biopsies, their clinical utility is significantly limited due to invasiveness, cost, inadequate sampling, and poor reproducibility.[7-11] "For-cause" biopsies, typically ordered in response to changes in clinical symptoms and declining renal function, for example rising SCr and proteinuria, share similar limitations and are often performed only after substantial allograft injury.[12] Subclinical rejection, without significant changes in renal function or proteinuria, is predicted by previous active rejection events and rising donor-specific antibody titers but requires biopsy for confirmation.[13]

SCr levels are commonly used to screen patients for AR and indicate when biopsy and histological evaluation of renal tissue are warranted.[8,14] Although easy to measure, SCr is a poor marker due to its low sensitivity and specificity. Furthermore, it is a lagging indicator of renal injury;[15] by the time SCr levels increase, the allograft may have undergone severe and irreversible damage.[6,16] Thus, there is a need for a simple, noninvasive, highly accurate assay that can detect ongoing AR.

Donor-derived cell-free DNA (dd-cfDNA) found in the plasma of transplant patients is a proven noninvasive biomarker for KT rejection.[2,9,14,17-19] dd-cfDNA has also been utilized in assessing graft function in other organ transplants (liver, heart, lung, and bone marrow).[2,20-26] We have previously demonstrated accurate quantification of cell-free DNA (cfDNA) mixture proportions using a single-nucleotide polymorphism (SNP)-based massively multiplexed polymerase chain reaction (SNP-mmPCR) methodology in the prenatal and multiplexed PCR (mPCR) methodology in oncology context.[27-30] Leveraging this technology, we have developed a noninvasive assay that estimates dd-cfDNA fraction (DF) in KT recipients by measuring the allele frequency at 13 962 SNPs chosen to maximize informative genotypes across ethnicities. A recent clinical validation study demonstrated the ability of this method to discriminate AR from nonrejection with a sensitivity of 88.7%, specificity of 72.6%, and area under the curve (AUC) of 0.87 using a DF cutoff of 1%.[14] In the current study, we analytically validate our clinical-grade next-generation sequencing (NGS) assay by determining the limit of blank (LoB), limit of detection (LoD), limit of quantitation (LoQ), linearity, precision (reproducibility and repeatability), and accuracy in measuring the DF in KT recipients in both related and unrelated donors.

MATERIALS AND METHODS

Samples

Plasma Mixture Samples

Whole blood samples (20 mL) were collected from healthy volunteers (n = 31) and transplant patients (n = 6) in Cell-Free DNA BCT tubes (Streck, Omaha, NE) in accordance with the Institutional Review Board (IRB)-approved protocol (Ethical and Independent IRB, Corte Madera, CA; approval number: IRB00007807, protocol number: 18-141) and the Declaration of Helsinki. All participants provided signed informed consents. Plasma (5–10 mL) was isolated from blood after centrifugation at 3220g for 30 minutes at 22°C and stored at –80°C. cfDNA was extracted using either Natera's in-house spin column-based chemistry for extraction (San Carlos, CA) or QIAamp Circulating Nucleic Acid Kit (Qiagen, Germatown, MD) and was used as either blanks (n = 15) for LoB or plasma mixture samples (n = 16). Plasma mixture samples were developed from 3 unrelated (1 male designated donor and 3 female designated recipient; StemExpress, Folsom, CA) and 6 related (3 mother-son pairs, 2 brother-sister pairs, and 1 uncle-niece pair) binary mixture samples. cfDNA concentration of plasma mixture samples was quantified using Quant-iT or Qubit dsDNA kits (Thermo Fisher, Carlsbad, CA).

Reference Samples (Cell-line Derived)

Reference samples were procured from SeraCare Life Sciences (Milford, MA) and were developed by mixing genomic DNA from 5 different cell lines to generate 3 binary female (recipient) to male (donor) reference mixtures (1 related and 2 unrelated) at specific targeted DFs (0%, 0.1%, 0.3%, 0.6%, 1.2%, 2.4%, 5%, 10%, and 15%). The DF in each reference mixture was verified by digital droplet polymerase chain reaction (ddPCR) by SeraCare. The genomic DNA mixtures were sheared by sonication and size selected to mimic expected cfDNA fragments of 160 base pairs by SeraCare. cfDNA concentration of the reference samples was quantified using Quant-iT or Qubit dsDNA kits.

Targeted Amplification, SNP Selection, Sequencing Data Analysis, and Quality Control

All samples were used as input for library preparation followed by targeted PCR amplification.[27] Targeted amplification was achieved by performing mmPCR as previously described, with a modification to the primer pool, which targeted 13 926 SNP positions (Figure 1).[29] Biallelic SNPs were selected on chromosomes 2, 13, 18, 21, 22, and X although only chromosomes 2, 13, 18, and 21 were included in the DF analysis. To ensure accurate DF estimate regardless of patient ethnicity, SNPs were required to have high minor allele frequency across the major ethnic groups as defined in the 1000 Genomes Project.[31]

The PCR amplicons generated after targeted amplification were barcoded and combined to generate 32-plex pools, which were sequenced using NGS technology (Illumina NextSeq 500 instrument, 50 cycles, single-end reads). Approximately 940 DNA copies were sequenced per locus. Sequenced reads were demultiplexed and mapped to the hg19 reference genome using Novoalign version 2.3.4 (http://www.novocraft.com/products/novoalign/). Bases with Phred quality score <30 and reads with mapping quality score <30 were filtered. Multiple quality checks (QCs) (cluster density, mapping rate, etc) were applied to the sequencing run, and each sample was confirmed to have the desired number of reads (8 million) after filtering. Any pool failing sequencing run QCs was resequenced.
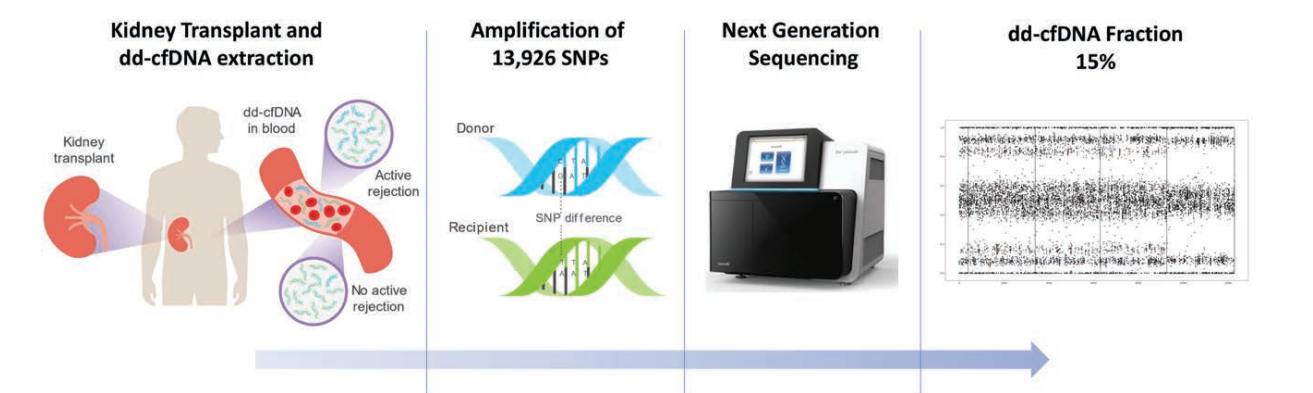
FIGURE 1. Workflow of a clinical grade next-generation sequencing assay. Donor-derived cfDNA is released from renal allograft into circulation; blood is drawn and centrifuged, and plasma is isolated. cfDNA is extracted from plasma samples and used for library preparation followed by targeted PCR amplification of 13 926 SNPs, performed using mmPCR. Amplicons are sequenced on a next-generation sequencer, and sequencing data are analyzed using a maximum likelihood estimate method to give a dd-cfDNA fraction, which is reported to the physician. cfDNA, cell-free DNA; dd-cfDNA, donor-derived cell-free DNA; mmPCR, massively multiplexed polymerase chain reaction; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

Any sample that failed to produce the necessary number of reads was removed from the analysis.

DF Calculation

For each sample, DF was estimated on the basis of the minor allele frequencies measured for all SNPs where the recipient was estimated to be homozygous. The DF calculation is a maximum likelihood estimate over a search range from 0.01% to 25% at increments of 0.01%. While the technology places no upper limit on the dynamic range of the assay, 25% was chosen for this study on the basis of the DF ranges observed in KT patients. Our approach did not include a separate donor sample. Donor genotype determination was not performed. Rather, a probability model was employed. No heuristic adjustment was needed for related donors because the algorithm does not incorporate prior assumptions regarding the level of genotype concordance between the recipient and the donor. Instead, the corresponding genotype inheritance constraints were incorporated into the donor genotype probability model when the donor and the recipient were related. This estimate mode was referred as "related estimate," and the unconstrained estimate was referred as "unrelated estimate."

Experimental Plan and Statistical Analysis

To evaluate analytical performance of the test, LoB, LoD, LoQ, linearity, precision, and accuracy were measured on the basis of Clinical and Laboratory Standards Institute (CLSI) guidelines (EP-17A2, EP05-A3).[32,33] Experimental design with sample type, input mass, DFs, and number of measurements for each study is listed in Table 1. All samples were tested with a minimum input amount of 15 ng and run in a minimum of triplicates, except for clinical samples, which were tested in duplicates (**Section S1:** Table S1, **SDC,** http://links.lww.com/TP/B701). Statistical analysis was performed using Python programming language

TABLE 1.

Experimental design

Performance metric	Sample type	Input mass, ng	Sample mixtures	dd-cfDNA fractions, %	Number of measurements	Total measurements
LoB	Reference (n = 5 blanks)	15, 30, 45	NA	NA	68	128
	Plasma (n = 15)	Variable	NA	NA	60	
LoD	Reference	15, 30, 45	1: related 2: unrelated	0.1, 0.3, 0.6	166 108	389
	Plasma mixtures (n = 16)	15 Variable	3: unrelated 6: related	0.1, 0.3, 0.6	60 55	
LoQ, linearity	Reference	15, 30, 45	1: related 2: unrelated	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10, 15	350 288	798
	Plasma mixtures (n = 16)	Variable	6: related	0.1, 0.3, 0.6, 1.2	64	
		15	3: unrelated	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10	96	
Accuracy	Reference	15, 30, 45	1: related 2: unrelated	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10, 15	350 288	638
Reproducibility	Reference	15, 30, 45	1: related 2: unrelated	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10	336 168	516
	Transplant patient (n = 6)	Variable	4: related 2: unrelated	Variable	12	
Repeatability	Reference	30	1: related	0.6, 2.4	128	128

dd-cfDNA, donor-derived cell-free DNA; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; NA, not applicable.

(Python Software Foundation, version 3.6, https://www.python.org/).

Limit of Blank

LoB was established using reference samples (blanks or single genome), obtained from SeraCare (n = 5), and plasma samples (n = 15) collected from healthy blood donors with no history of organ transplant or recent blood transfusion (Table 1). Reference samples were prepared at different targeted library input amounts to mimic the expected range of cfDNA yields achieved from 20mL blood collections. Plasma samples were used at their unadjusted concentrations to reflect the variable cfDNA yields typical from real samples. In compliance with Clinical and Laboratory Standards Institute guidelines (EP-17A2),[32] samples were tested in triplicate on 3 different days with 2 different sequencing reagent lots that consisted of at least 60 measurements per lot.

LoB is defined as the empirical 95th percentile value measured from a set of blank (no-analyte) samples. The calculation is performed for the reference samples and plasma samples from each reagent lot (lots 1 and 2) and for each DF estimation method, that is, unrelated and related. For each estimation method, the final LoB was the maximum of the 2 per-lot results.

Limit of Detection and Limit of Quantitation

LoD and LoQ were measured using both reference samples and plasma mixture samples from healthy volunteers at different cfDNA input amounts. LoD was measured at the three lowest DFs by 2 operators on different days using different reagent lots and sequencing instruments. LoQ analysis was performed on the same samples as LoD with additional replicates at higher DFs (Table 1).

LoD was calculated following the parametric estimate method specified in EP-17A2,[32] which computes LoD by adding an standard deviation (SD) term to the LoB. LoDs for reference samples and plasma mixture samples for each reagent lot were calculated for each DF estimation method by combining the corresponding LoBs and SD measurements. Similar to LoB, for each estimation method, the final LoD value was calculated using the maximum value of lots 1 and 2, calculated with the corresponding method. Furthermore, LoDs were also calculated separately for each DF estimation method for plasma mixture samples and reference samples, at each input amount.

An appropriate LoQ assessment was selected on the basis of the quantification requirements of the test process. LoQ is defined as the lowest DF at which a sufficient relative measurement precision is achieved, lower bounded by the LoD. We defined sufficient relative measurement precision as 20% coefficient of variation (CV). The relationship between DF and its CV was modeled as CV = $a + b \times \exp(-c \times DF)$, where the model parameters a, b, and c were estimated from the data using a nonlinear least-squares procedure. The CV model (described by parameters a, b, and c) was estimated for each DF estimate method and used to evaluate the LoQ criterion mentioned previously. This model-based approach requires inclusion of higher DF measurements for the LoQ assessment to ensure convergence to an appropriate constant value at high DF. In line with the aforementioned LoD calculation, LoQs for

reference samples and plasma mixture samples were calculated for each reagent lot and DF estimation method. The final LoQ value was calculated from the maximum of the values of lots 1 and 2, calculated using the corresponding method. LoQs were also calculated for each DF estimation method separately for plasma mixture samples and for reference samples at each input amount.

Linearity and Accuracy

Linearity and accuracy were measured using the same sample set as described for LoQ, with the accuracy measurement restricted to reference samples only. Linearity was evaluated on the basis of the R^2 value produced by a standard linear regression analysis of the relationship between measured DF and targeted mixture fractions for each DF estimation method. Linearity was evaluated for both reference and plasma mixture samples separately for each DF estimation method. Accuracy was evaluated on the basis of the linear regression analysis of the relationship between measured DF and the orthogonal ddPCR measurement for each DF estimation method.

Precision

Precision was measured by testing reproducibility (interrun) and repeatability (intrarun) across reference and transplant samples. Matched blood draws (4 tubes per patient) from transplant recipients were run in duplicates and were evaluated for reproducibility in clinical samples. Reproducibility samples were processed by 2 different operators on 8 different days (24 runs across 23 days) with 3 reagent lots and 17 sequencing instruments. Repeatability was determined by measuring variability between technical replicates of samples measured under similar conditions. One related (mother-son) reference mixture at 2 DFs was assayed by a single operator, reagent lot, and instrument.

Repeatability, defined as the CV measured across the set of replicates at a single targeted DF, under matched conditions was calculated once at 0.6% and once at 2.4% DF. Reproducibility was also measured using CV, calculated separately for each combination of DNA input amount and mixture fraction.

RESULTS

Limit of Blank

LoB was calculated using 64 measurements from lots 1 and 2 each. The LoB was found to be 0.11% for the unrelated donor estimate and 0.23% for the related donor estimate. Evaluation of plasma sample measurements only (60 measurements total, combined across both lots) resulted in LoB of 0.04% (unrelated) and 0.08% (related), suggesting a significantly lower LoB in plasma samples when compared with that of reference samples. Figure 2 shows histograms of the relevant DF measurements broken down by method and lot (**Section S2:** Figure S1 and Tables S2 and S3, **SDC,** http://links.lww.com/TP/B701).

LoD and LoQ

LoD was calculated from 168 and 220 measurements from unrelated and related samples, respectively, resulting in LoD of 0.15% (unrelated) and 0.29% (related). One

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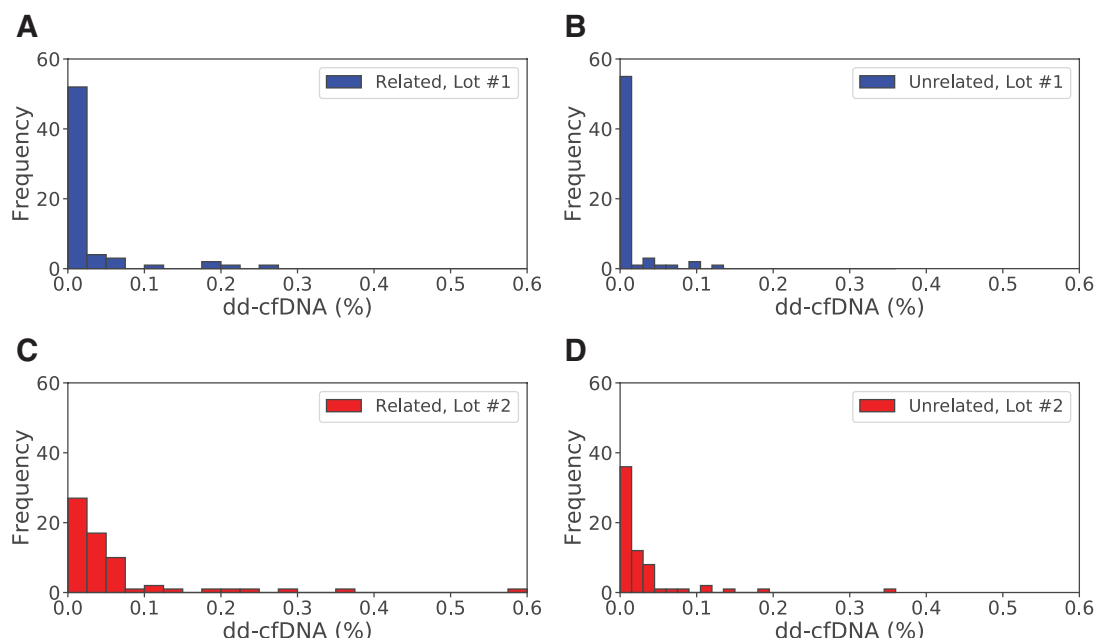
FIGURE 2. Histograms of measured dd-cfDNA for LoB analysis. A, Related method, lot 1. B, Unrelated method, lot 1. C, Related method, lot 2. D, Unrelated method, lot 2. dd-cfDNA, donor-derived cell-free DNA; LoB, limit of blank.

sample was excluded from the analysis due to failed QC. The difference in LoD for related versus unrelated donors was approximately equal to the difference in corresponding LoB, meaning that the measurement variance near the LoD was approximately the same in the two methods. The LoD was not significantly different at the different DNA input amounts (**Section S3:** Figures S2 and S3 and Table S4, **SDC,** http://links.lww.com/TP/B701). Restricting the measurements to plasma mixture samples yielded lower estimated LoD: 0.05% (unrelated) and 0.11% (related), although the number of measurements performed was less (54, related and 60, unrelated) than suggested by the guidelines (**Section S3:** Table S5, SDC, http://links.lww.com/TP/B701).

LoQ was calculated from 381 and 412 measurements from unrelated and related samples, respectively, after exclusion of 5 samples that failed QC. Upper LoQ is the largest DF tested, which is 15%. The empirical CVs were all found to be <20% at each targeted DF, including reference and plasma mixture samples. Empirical CVs and the resulting parametric models are shown in Figure 3. The modeled CVs were also found to be <20% for all DFs greater than or equal to the LoD. Thus, the LoQ is equal to the LoD for all relevant scenarios. This was observed to be true when the analysis was restricted to plasma mixture samples only, as well as to reference samples only, at each input amount (**Section S4:** Figures S4 and S5 and Table S6, **SDC,** http://links.lww.com/TP/B701). For ease of reference, Table 2 summarizes the results of LoB, LoD, and LoQ.

Linearity, Accuracy, and Precision

Linearity was measured from 381 unrelated and 412 related samples, after removal of 5 samples that failed QC. Accuracy was measured from the subset of these (reference samples) for which ddPCR-measured DF was available as a reference (285 unrelated and 349 related

samples), excluding 4 that failed QC. Linearity was also evaluated for 6 clinical transplant patient samples using 12 measurements, all of which passed QC. The individual measurements and linear regression lines are shown in Figure 4 (linearity) and Figure 5 (accuracy). Figure 6 shows the measured DFs from lot 2 plotted against those from lot 1 and the linear regression line for clinical transplant patient samples.

Linearity was measured by linear regression against the targeted DF, and accuracy was measured by linear regression against the ddPCR-measured DF. Linearity for clinical transplant patient samples was measured by a linear regression of the measured DF from lot 2 plotted against that from lot 1. The linear regression results are given in Table 3. The DF measurement was observed to be highly linear (R^2 >0.99 in all models) and accurate (slope approximately 1, intercept approximately 0) with no significant difference between related and unrelated donors. Linearity and accuracy analyses restricted to plasma mixture and reference samples only are provided in the Supplemental Material and Methods (**Section S5:** Figures S6 and S7 and Table S7; Section S6: Figure S8 and Table S8, **SDC,** http://links.lww.com/TP/B701).

Precision was estimated by evaluating CV in 2 scenarios: repeatability within a single set of conditions and reproducibility across a varied set of conditions. CV calculations combined samples with related and unrelated donors. Repeatability was measured at 2 targeted DFs (0.6% and 2.4%), each using 64 reference sample measurements with all samples passing QC.

Per-input reproducibility was calculated by using 498 measurements, excluding 6 samples that failed QC. Per-lot reproducibility was calculated from a subset of the aforementioned samples, whose cardinality was 374, excluding 4 samples that failed QC. Reproducibility of DF in clinical transplant patient samples was calculated using the aforementioned 12 measurements. The estimated CVs,
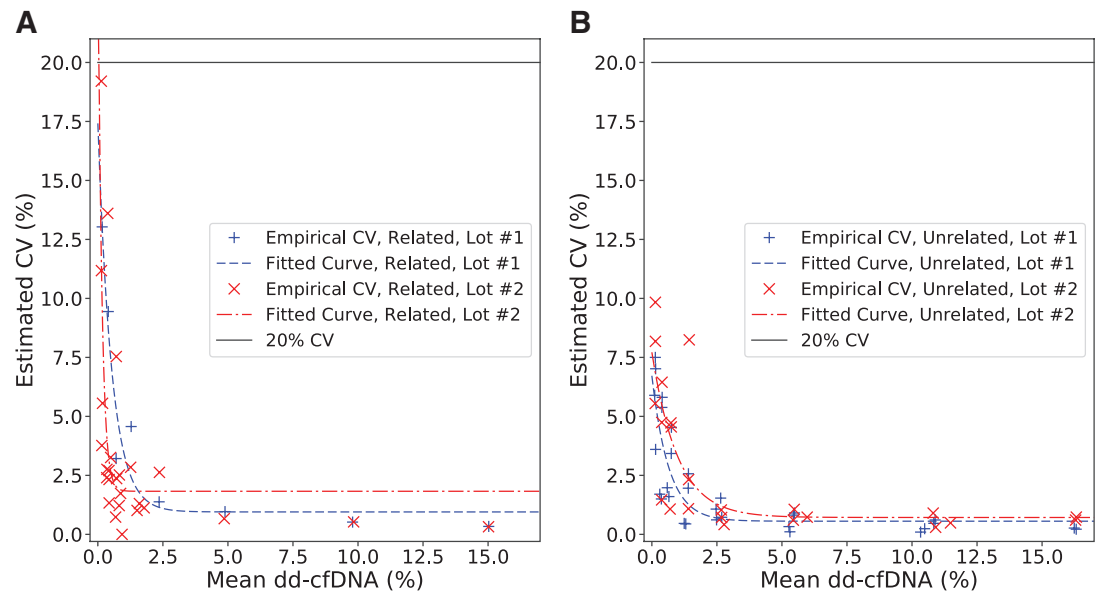
FIGURE 3. Measured CV values (%) as a function of the corresponding empirical means (%) for LoQ analysis. A, Related samples. B, Unrelated samples. CV, coefficient of variation; LoQ, limit of quantitation.

along with 95% confidence interval (CI), are provided in Table 4. Finally, for clinical transplant patient samples, 100% concordance of clinical calls was observed (95% CI: 54.07%–100%) between replicates (**Section S7: Figure S9, SDC,** http://links.lww.com/TP/B701).

DISCUSSION

Early detection of rejection in KT recipients holds promise for improved outcomes, but this goal remains unmet due to the unavailability of accurate, noninvasive methods to detect allograft rejection before substantial injury has occurred. Given the limitations associated with current allograft monitoring practices, most notably SCr and biopsy, there exists an opportunity to develop better tools for early detection of allograft rejection.

Several studies have shown the clinical relevance of noninvasive dd-cfDNA assays, which gives an assessment of the likelihood of AR in recipients of KT.[14,18] In 2017, Bloom et al.[18] using a different targeted NGS approach, correlated plasma DF and rejection status in 107 biopsy-matched specimens, demonstrating a significant difference

in median DF between cases with AR (1.6%) and nonrejection (0.3%; $P < 0.001$) using a predefined cutoff of >1%, with sensitivity and specificity of 59% and 85%, respectively. In a more recent study, Sigdel et al.[14] evaluated DF in 217 biopsy-matched plasma samples and showed the superior performance of dd-cfDNA in differentiating AR (including subclinical rejection) from nonrejection (including stable, borderline, and other injury cases) compared with estimated glomerular filtration rate, with a sensitivity of 88.7% versus 67.7%, specificity of 72.6% versus 65.3%, and AUCs of 0.87 versus 0.74, respectively. The study showed similar performance of the dd-cfDNA assay in protocol and for-cause biopsies, as well as the ability to detect both antibody-mediated and T cell-mediated rejection.[14] The assay did not need prior genotype information and was robust to different donor-recipient relationships.

The current study addresses the analytical validity of the DF quantification method used in Sigdel et al. Patients with DF of ≥1% are classified as "at increased risk of organ rejection";[14] analytical performance should be interpreted in the context of accurately classifying a sample with respect to this threshold. In this study, LoD and LoQ were shown to be 0.15% for unrelated donors and 0.29% for related donors, indicating an ability to accurately quantify DF at a level significantly lower than the classification threshold. When analysis was restricted to plasma mixture samples, which are more reflective of clinical samples, the LoD and LoQ were observed to be significantly lower (0.05% LoD and LoQ for unrelated donors). This difference can be partly attributed to the significantly higher per-base insert error rate in reference samples compared with that in plasma samples in the LoB calculation (0.001355 versus 0.001170, $P < 0.0001$, independent t-test). Contrived reference samples are commonly substituted for plasma samples in analytical testing, and this difference in the error rate is negligible in evaluations other than LoB. Analytical performance of a different SNP-based assay[2] for measuring DF in KT recipients reported similar LoD and LoQ (0.15%, and 0.2%, respectively) but did

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TABLE 2.

LoB, LoD, and LoQ values for each estimation method and various sample types

Performance metric, estimation method	Combined samples,[a] %	Plasma or plasma mixture samples only, %
LoB, related	0.23	0.08
LoB, unrelated	0.11	0.04
LoD, related	0.29	0.11
LoD, unrelated	0.15	0.05
LoQ, related	0.29	0.11
LoQ, unrelated	0.15	0.05

[a]Combined samples include reference and plasma samples for LoB calculation and reference and plasma mixture samples for LoD and LoQ calculation.
LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation.
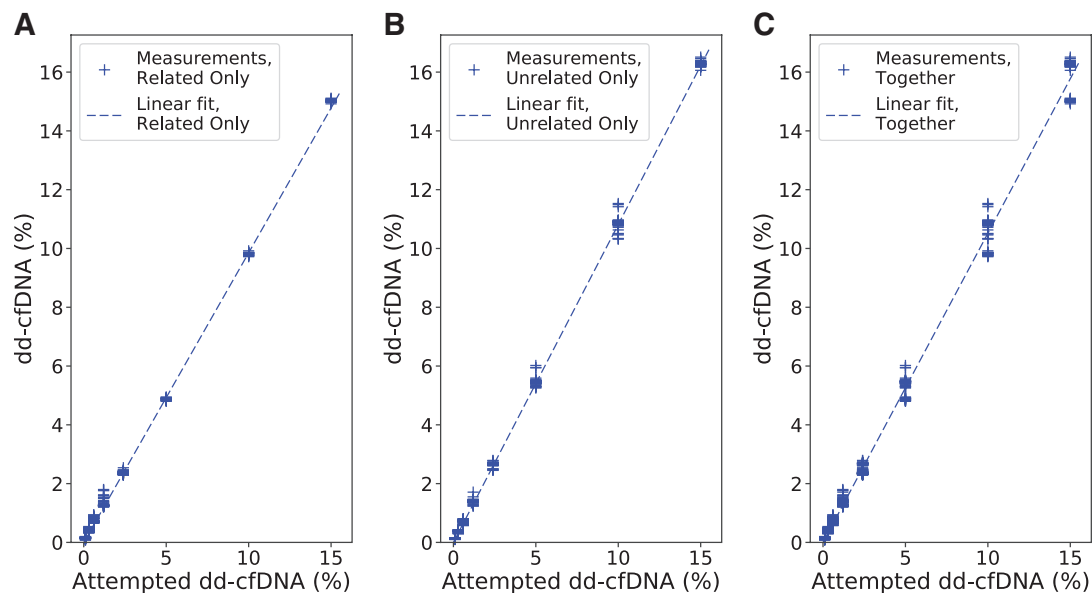
FIGURE 4. Measured dd-cfDNA as a function of the corresponding attempted spike levels, along with the calculated linear fit, for linearity analysis. A, Related only. B, Unrelated only. C, Related and unrelated cases together. dd-cfDNA, donor-derived cell-free DNA.

not report any distinction between reference and plasma samples although they were both evaluated.

The current method was also confirmed to have high accuracy on the basis of linear regression analysis, comparing measurements on >600 samples to an orthogonal DF measurement, ddPCR. Performance was evaluated with respect to a range of DNA input masses, selected to represent the expected distribution of cfDNA yields achieved from the clinical protocol-specified 20mL blood collections. No detectable performance difference was observed at different DNA input levels. Precision studies showed that the DF measurement was stable across intrarun and interrun replicates, across multiple lots of critical reagents, and between repeat (concurrent) blood draws from the

same patient. This indicates that the test is appropriate for large-scale implementation in a clinical laboratory setting.

This assay achieved superior measurement precision close to the classification range, as compared to a previously published assay,[2] with a CV of 1.85% versus 9.2% (within run, at 0.6% DF) and a CV of 1.99% (across runs, at 45 ng input) versus 4.5% (across runs, at 60 ng input). The approximately 5-fold difference in CV measured within run at a DF relatively close to the classification threshold indicates that this assay has improved precision; taken in combination with the higher AUC demonstrated previously,[14] this suggests that the higher precision may have a positive impact on clinical accuracy. Several factors may account for this improvement in performance. The library preparation step reduces variability
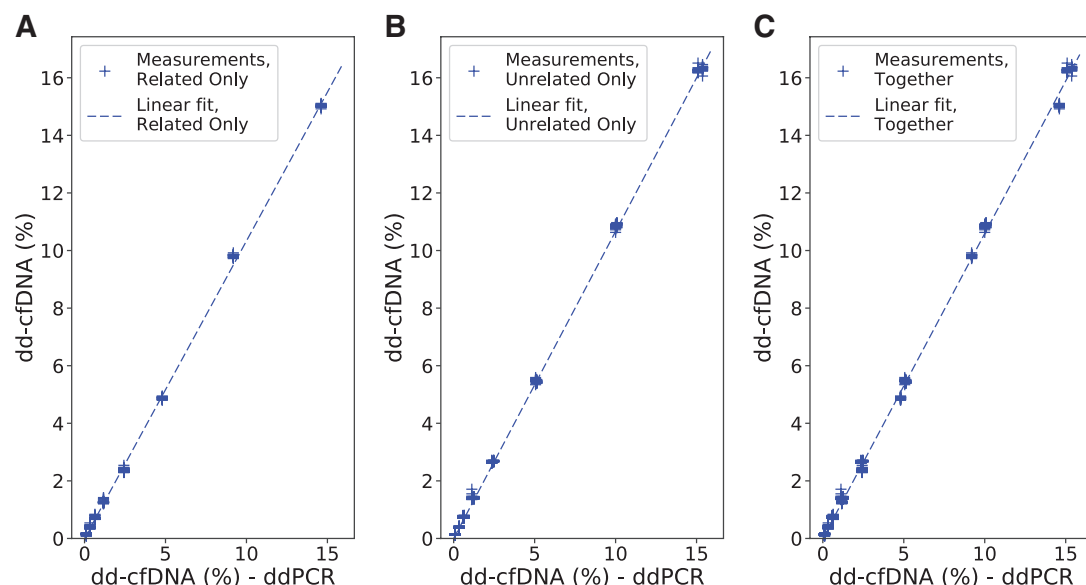
FIGURE 5. Measured dd-cfDNA as a function of the corresponding ddPCR values, along with the calculated linear fit for accuracy analysis. A, Related only. (b) Unrelated only. (c) Related and unrelated cases together. dd-cfDNA, donor-derived cell-free DNA; ddPCR, digital droplet polymerase chain reaction.
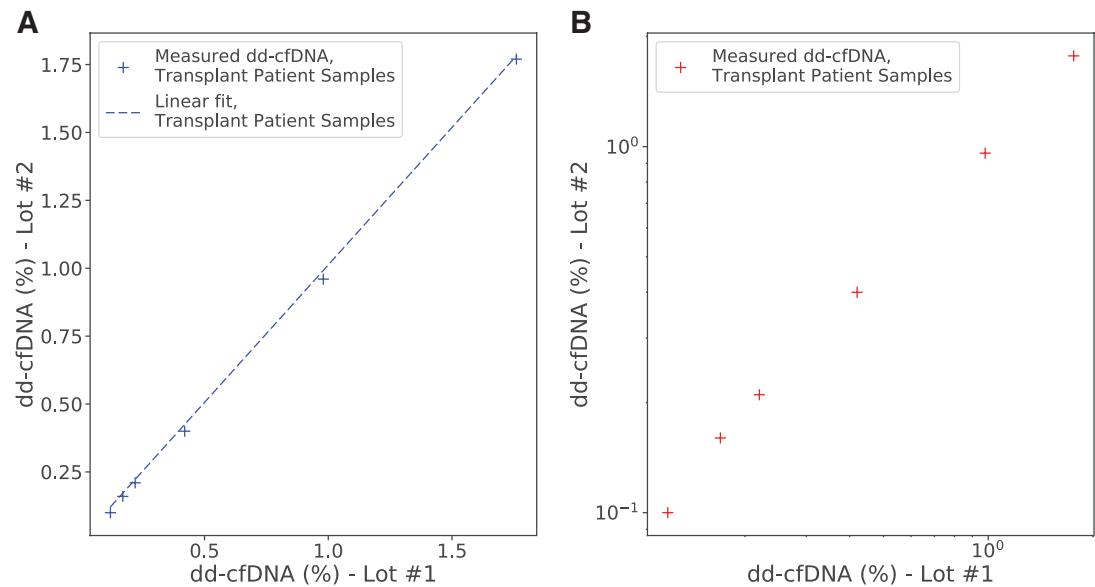
FIGURE 6. Measured dd-cfDNA from lot 2 as a function of the values from lot 1. A, On linear scale, along with the calculated linear fit. B, On log-log scale. dd-cfDNA, donor-derived cell-free DNA.

caused by the long DNA fragments. Also, the very large number of targeted SNPs (13926) and the probability model for donor genotypes enable an accurate DF estimation independent of the degree of relationship between the donor and the recipient. This is important due to the concern that the higher rate of genotype concordance (implying a lower rate of informative genotypes) in a related donor scenario might limit the accuracy of DF estimates. This study tested a large number of mixture sample replicates from mother-child and other related donor pairings and showed an LoB that was higher in related donor pairs, which led to correspondingly higher LoD, although these limits were still substantially below the assay's clinical threshold. All the other metrics, including linearity, accuracy, and the various precision metrics, were equivalent between related and unrelated donor pairs, showing that the quantitative performance of the test is not meaningfully impacted by the reduced number of informative genotypes. The previously published method addressed this concern through in silico estimates but did not confirm test performance on reference samples or plasma mixture samples from related individuals.[2]

Several other methods have been used to measure dd-cfDNA levels in transplant recipients. Beck et al[20] described a fast, inexpensive ddPCR method and demonstrated its use in heart, liver, and KT patients; the CV was shown to range from 4% to 14%, and precision was not measured <2%, which is above the cutoff used in the current study. De Vlaminck et al.[21,24] described a shotgun NGS method, which was shown to detect mild and moderate-to-severe rejection events with an AUC of 0.75; the method required prior genotyping of the donor and recipient before transplant. Sharon et al.[25] recently described another shotgun NGS method that overcomes the need for prior genotyping of the donor, although not the recipient, that estimated dd-cfDNA in both related and unrelated donor-recipients. None of these methods have been validated for clinical use. The SNP-based mmPCR assay described in the current study does not require prior genotyping of either donor or recipient and detects DF with a high precision (a CV of <2%), irrespective of the donor-recipient relationship. Although this study has only validated the assay for use in KT, we expect that it will show clinical value in other organ transplants such as heart, liver, and bone marrow.

CONCLUSIONS

With an unacceptably high rate of allograft rejection in KT recipients, a paradigm shift in the management of renal

Linear regression results for linearity and accuracy, including 95% CI

Performance metric, data set	Linearity and accuracy parameters		
	Slope	Intercept	R^2
Accuracy, combined	1.0591 (0.9763, 1.1418)	0.0001 (−0.0045, 0.0047)	0.9988 (0.9987, 0.9990)
Accuracy, related	1.0333 (0.9241, 1.1425)	−0.0001 (−0.0047, 0.0046)	0.9989 (0.9986, 0.9990)
Accuracy, unrelated	1.0664 (0.9416, 1.1912)	0.0008 (−0.0076, 0.0092)	0.9997 (0.9997, 0.9998)
Linearity, combined	1.0516 (0.9781, 1.1251)	0.0004 (−0.0033, 0.0042)	0.9968 (0.9964, 0.9972)
Linearity, related	0.9852 (0.8895, 1.0809)	0.0008 (−0.0031, 0.0047)	0.9991 (0.9989, 0.9992)
Linearity, unrelated	1.0813 (0.9721, 1.1906)	0.0006 (−0.0060, 0.0071)	0.9995 (0.9994, 0.9996)
Linearity, transplant patient samples	1.0125 (−0.3932, 2.4183)	−0.0002 (−0.0121, 0.0117)	0.9998 (0.9984, 1.0000)

CI, confidence interval.

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TABLE 4.

Estimated CVs, including 95% CI, for repeatability and reproducibility for different scenarios

Performance metric	CV, %
Repeatability, 0.6% DF	1.85 (1.34, 2.73)
Repeatability, 2.4% DF	1.22 (0.88, 1.80)
Per-input reproducibility, 15 ng	3.10 (1.58, 4.37)
Per-input reproducibility, 30 ng	3.07 (1.42, 4.50)
Per-input reproducibility, 45 ng	1.99 (1.10, 2.75)
Per-lot reproducibility, lot 1	3.99 (2.42, 5.41)
Per-lot reproducibility, lot 2	4.44 (2.69, 6.02)
Reproducibility, transplant patient samples	4.29 (0.65, 6.86)

CI, confidence interval; CV, coefficient of variation; DF, dd-cfDNA fraction.

allograft health is overdue. Routine measurement of dd-cfDNA, with its ability to detect rejection early and noninvasively, represents a key strategy to improve clinical outcomes. Ongoing registry studies seek to demonstrate the efficacy of dd-cfDNA to detect allograft rejection, and its corresponding utility for optimizing biopsy use, immunosuppressive regimen, and improving graft survival rates. This study demonstrates the analytical validity of an accurate, noninvasive SNP-based dd-cfDNA assay. Taken alongside the clinical validation of this assay, demonstrated by Sigdel et al,[14] this heralds a new diagnostic tool for nephrologists with the promise of better patient management and outcomes.

ACKNOWLEDGMENTS

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Guidelines for the Diagnosis of Antibody-Mediated Rejection in Pancreas Allografts—Updated Banff Grading Schema

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The first Banff proposal for the diagnosis of pancreas rejection (Am J Transplant 2008; 8: 237) dealt primarily with the diagnosis of acute T-cell-mediated rejection (ACMR), while only tentatively addressing issues pertaining to antibody-mediated rejection (AMR). This document presents comprehensive guidelines for the diagnosis of AMR, first proposed at the 10th Banff Conference on Allograft Pathology and refined by a broad-based multidisciplinary panel. Pancreatic AMR is best identified by a combination of serological and immunohistopathological findings consisting of (i) identification of circulating donor-specific antibodies, and histopathological data including (ii) morphological evidence of microvascular tissue injury and (iii) C4d staining in interacinar capillaries. *Acute AMR* is diagnosed conclusively if these three elements are present,

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whereas a diagnosis of *suspicious for AMR* is rendered if only two elements are identified. The identification of only one diagnostic element is not sufficient for the diagnosis of AMR but should prompt heightened clinical vigilance. AMR and ACMR may coexist, and should be recognized and graded independently. This proposal is based on our current knowledge of the pathogenesis of pancreas rejection and currently available tools for diagnosis. A systematized clinicopathological approach to AMR is essential for the development and assessment of much needed therapeutic interventions.

Introduction

Over the past three decades, refinements in surgical techniques and greater understanding of the histopathological features of allograft rejection have been achieved in vascularized pancreas transplantation (1–9). Moreover, major pharmacological advances have been made in the prevention and treatment of classical acute T-cell-mediated allograft rejection (ACMR) leading to higher expectations for improved short- and long-term outcomes. Unfortunately, in spite of a reduction in the rates of early acute rejection, long-term outcomes have not improved markedly, because some subsets of patients continued to lose their grafts to intractable rejection or to slow but inexorable progression to graft fibrosis (10,11).

In recent years an increasing number of cases of pancreas allograft dysfunction and loss attributed to antibody-mediated allograft rejection (AMR) have been reported in the literature (6,11–15). AMR which does not respond to standard treatments for ACMR is caused by antibodies directed against donor-specific human leukocyte antigen (HLA) molecules or other cellular antigens (12,16,17). AMR can result from a strong anamnestic antibody response to previous antigenic exposure (i.e. retransplantation and pregnancy) or from *de novo* development of donor-specific antibody (DSA; Ref. 11). The development of AMR was documented in a pancreas-transplant recipient who was presensitized after two previous islet transplantations (18) and has also been reported in association with viral infections (19,20).

AMR causes graft failure through acute and/or chronic immunoglobulin and complement induced microvascular injury and remodeling that eventually leads to graft fibrosis (21,22). It has been postulated that chronic AMR is the single most important factor limiting long-term graft survival in solid organ transplantation (21,23,24). The interplay between AMR and autoimmunity is currently unknown (25), but anti-HLA DSA and AMR have also been reported in association with recurrence of autoimmune diabetes mellitus (14).

Distribution of HLA Class I and Class II in pancreas tissue

Major histocompatibility complex (MHC) disparities have been associated with an increased risk of humoral rejection and graft loss (26), but in practice minimal emphasis is placed on HLA matching in simultaneous kidney-pancreas transplantation. HLA matching may have a greater role in solitary pancreas transplantation, in which the incidences of ACMR and immunological graft loss are inherently higher (1).

The normal pancreas expresses MHC Classes I and II differently in the exocrine and endocrine components (27–29). Expression is altered in inflammatory conditions, including ACMR, which is typically associated with aberrant expression of Class I and Class II antigens (30–33). Similarly, with the development of diabetes mellitus there is hyperexpression of Class I antigens and aberrant expression of Class II antigens in the endocrine islets (34; Table 1).

Considerations on DSA testing

Prevention, diagnosis and treatment of AMR require monitoring for the presence of circulating DSA (35). It is recommended that routine antibody monitoring be performed at regular intervals after transplantation, as well as at the time of biopsy, and whenever rejection is suspected (36). Specific clinical settings may warrant development and implementation of protocols tailored to individual patients (i.e. desensitization protocols, weaning of immunosuppression, etc.) (37).

In recent years, marked improvements in the sensitivity and specificity for detecting alloantibodies have led to ongoing assessment of the clinical relevance of anti-HLA antibody levels, specificities and the significance of antibodies to non-HLA antigens (e.g. MHC class I–related chain A [MICA], auto antigens) and to non-AB-DR HLA antigens (37–39). Although an earlier study found a strong association between DSA to MHC Class II and chronic allograft rejection/graft loss (26), subsequent studies have not found

Table 1: Class I and II HLA expression in normal and abnormal pancreas tissue*

Cell type	Normal pancreas histology sections		Tissue culture–inflammatory milieu (ß IFN, γ IFN and IL2)		Diabetes mellitus (DM)	
	Class I	Class II	Class I	Class II	Class I	Class II
Acinar cells	−	−	+ Aberrant expression	+ Aberrant expression	n/a	n/a
Ductal cells	++	−	++	+ Aberrant expression	n/a	n/a
Islet cells	+/− (Weak)	−	++ Hyperexpression	++ Aberrant expression	++ all islet cells hyperexpression (with insulitis -early DM)	+ ß cells aberrant expression, +/− insulitis
Capillary endothelium	++	++	n/a	n/a	n/a	n/a
Large vessel endothelium	++	Variable	n/a	n/a	n/a	n/a

*Based on Refs. 10–17.
n/a = data not available.

significant clinicopathological differences between DSA to Class I and Class II antigens (6,12,15). Antibodies to MICA were associated with histopathological features of AMR in the pancreas in one series (15)

Although there has been remarkable progress to date, much work remains to be done in the area of histocompatibility and immunogenetics in order to better understand and treat AMR (see section later on future directions).

C4d Staining

Background

Circulating DSA directed against endothelial cells leads to widespread activation of the complement and coagulation cascades in the vascular walls with consequent mobilization of a variety of inflammatory mediators. Demonstration of immunoglobulins and active complement components in the microvasculature has proven difficult due to the rapid turnover and degradation of the various products (40). In contrast, the complement fragment C4d which is generated through the classical (antibody-induced) activation pathway is resistant to shedding and degradation and remains detectable in the vessel walls for at least several days following the initial immunological event (17,40,41).

Multiple studies have demonstrated that C4d staining in renal biopsies performed for allograft dysfunction is predictive of poorer graft outcomes and helps identify patients with AMR (16). In pancreas allograft biopsies, C4d staining is typically absent in cases of pure ACMR or in protocol biopsies from well-functioning grafts (6,12).

More recently, it has been emphasized that C4d staining alone lacks enough sensitivity and specificity to be used as an unequivocal marker for the presence or absence of renal AMR, especially late posttransplant (42,43). However, microvascular inflammation/injury with concurrent detection of circulating DSA can identify AMR independently of positive C4d staining (22,44). For practical purposes, it is generally agreed that the clinical diagnosis of AMR is best achieved with a combination of careful histological evaluation, including C4d staining and correlation with concurrent DSA evaluation (9,45).

Technical aspects and interpretation

There is general consensus that C4d staining should be performed in pancreas allograft biopsies in addition to the standard set of slides and stains (Hematoxylin and Eosin [H&E] ×3 and Masson's trichrome stain; Ref. 9). Both immunohistochemical and immunofluorescence C4d stains are adequate for diagnosis and yield a similar staining pattern in interacinar capillaries (IAC; Figure 1). In renal and cardiac allograft biopsies, the immunofluorescence technique has been reported to yield stronger staining compared to the immunohistochemical method but the difference was not considered significant for clinical purposes (45,46). Comparison of the two methods in pancreas allograft biopsies, showed that with the immunofluorescence technique the estimated areas of lobular IAC staining were 10–50% larger than with the immunohistochemical method (E. Rangel, D. U. Kim and P. Revelo, ongoing data collection). Based on these preliminary data and the earlier work by Torrealba et al. (6), it is recommended that the threshold for C4d positivity in pancreas-allograft biopsies should remain at ≥5% (9), until additional data become available. When immunohistochemical staining is used, a low threshold is preferable, considering that several studies in the kidney

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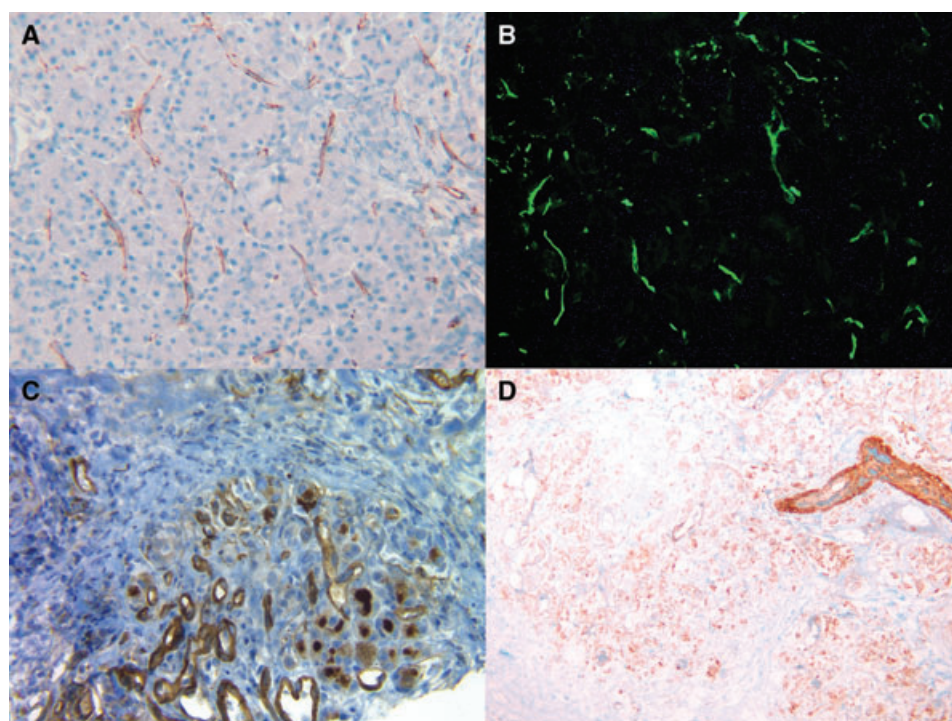
Figure 1: C4d staining in pancreas allografts. (A and B) Immunohistochemical and immunofluorescence C4d staining demonstrates comparable interacinar capillary staining (Courtesy: Dr. Revelo). (C) Atrophic lobule in chronic active AMR shows strong C4d positivity in residual interacinar capillaries. The acinar component is atrophic. Note "lobular" arrangement of the staining capillaries. (D) C4d staining in severe acute AMR. Due to extensive parenchymal necrosis there is nonspecific background staining with very rare recognizable positive interacinar capillaries. A thrombosed necrotic artery shows positive staining in its wall and contents.

have shown that both focal and diffuse C4d staining were associated with poorer graft outcomes (17,47). In a patient with pancreatic AMR, semiquantitive evaluation of intensity and extent of C4d staining in serial biopsies was found to correlate with DSA levels (S. Seshan, personal observation).

C4d staining in parenchymal-IAC is to be reported semiquantitatively based on the extent of exocrine lobular biopsy surface staining, as follows: Negative <5%, Focal 5–50% and Diffuse >50%. Only linear or granular staining along the IAC correlates with the presence of circulating DSA (6,12). In contrast, staining in other tissue components such as the endothelium of larger vessels including veins and arteries, the interstitial or septal connective tissue or the peripancreatic soft tissues is considered nonspecific (6). In biopsies with chronic active AMR (see later), the lobular architecture is expected to be disrupted by interstitial fibrosis and acinar atrophy and it may be more difficult to identify the IAC. On the other hand, despite the sclerosing architectural changes, C4d positivity typically remains in residual capillary vessels often with partial preservation of the lobular arrangement (Figure 1C).

In severe AMR with extensive parenchymal necrosis, most of the IAC staining could be lost. In contrast, strong C4d staining is typically found in the necrotic vascular walls (Figure 1D). Correlation with DSA studies is strongly recommended in this setting.

Clinicopathological Spectrum of AMR in Pancreas Allografts

Hyperacute rejection

The inescapable effects of preformed antidonor antibodies leading to "hyperacute rejection" and immediate graft destruction were identified early in the history of solid organ transplantation as a strong immunological barrier to successful engraftment. With respect to pancreas transplantation, the recognition of *hyperacute rejection* has been obscured by the high propensity of this organ for early graft thrombosis that may or may not be related to rejection (2,48). Sibley (49) first described a case of hyperacute rejection, in a patient with a negative pretransplant cross match but high-level panel-reactive antibody (PRA) when retested after the removal of the thrombosed organ. Similar cases were described later, with graft loss occurring either immediately (*hyperacute rejection*) or within hours posttransplantation. As in the case reported by Sibley (49), circulating DSA were identified retrospectively, in the setting of an initially negative cytotoxic cross match (2).

Pathological findings in biopsies with severe, irreversible AMR correspond to those observed in experimental models of hyperacute rejection (50). The earliest changes occur within minutes of revascularization and consist of edema, congestion, spotty acinar cell injury (i.e. vacuolization, degranulation and necrosis) and capillary and venular neutrophilic margination. Progressive graft destruction occurs within a few hours and is characterized by confluent foci of

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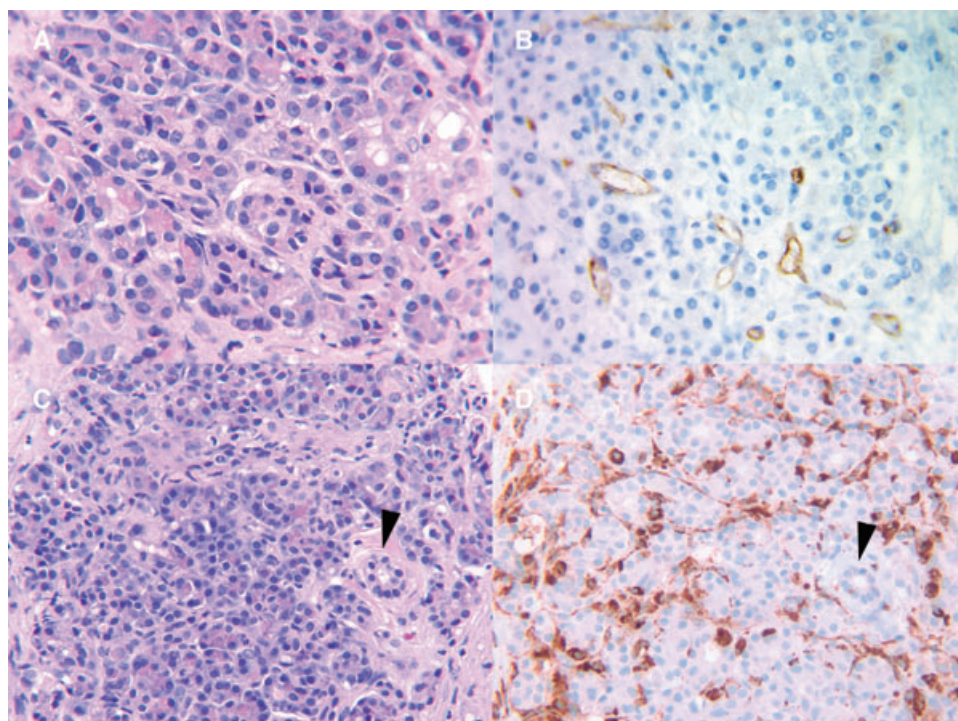
Figure 2: Mild acute AMR. (A) Exocrine area with very subtle interstitial, interacinar inflammation and (B) scattered IAC outlined with the C4d stain. (C) Preserved lobular/acinar architecture and mild mononuclear infiltrates that are underestimated on the H&E stain. (D) CD68 stain for macrophages in the same area of the biopsy as (C), demonstrates the extent of the infiltrates (arrowheads in C and D mark a small duct for orientation).

hemorrhagic necrosis in acini, islets and ducts, with prominent neutrophilic infiltrates and widespread fibrinoid vascular necrosis and thrombosis (50). Immunoglobulin (i.e. IgG) and complement deposition including C4d staining are found throughout the graft vasculature (2,9).

Acute AMR

Awareness of acute AMR in pancreas allografts was heightened by the characterization of this entity in kidney transplants (16) and recognition of the negative impact of circulating DSA on both short- and long-term pancreas graft survival rates (6,11,12,51–54).

In recent years, a considerable number of studies have reported the clinical and pathological findings of acute AMR in pancreas allografts, which typically presents with graft dysfunction. In the studies of de Kort (12), Rangel et al. (15) and Torrealba et al. (6) exocrine abnormalities (increase in serum amylase/lipase or decrease in urine amylase levels) represented the most common indication for allograft biopsy (55–70%) followed by combined exocrine and endocrine abnormalities (15–20%). Isolated endocrine dysfunction (hyperglycemia) was a relatively rare indication for allograft biopsy (6–8%). The unusual association between AMR and pancreatic panniculitis was recently reported (55).

Although approximately 75% of cases of acute AMR were diagnosed in the first 6 months posttransplantation, late occurring cases were not unusual (average 248 days, median 79 days, range 1–3331 days; Refs. 6,11–14,52), clearly

paralleling the clinico-pathological spectrum described with AMR in renal allografts (17). ACMR and acute AMR could not be distinguished from each other on clinical grounds, stressing the importance of DSA monitoring and biopsy evaluation (15)

The morphological findings in acute AMR may consist of various degrees of inflammation and tissue injury as detailed below:

- Acinar/interacinar inflammation
  In its earlier, milder forms acute AMR presents with overall preservation of the architecture and mild interacinar monocytic and/or neutrophilic infiltrates associated with subtle spotty acinar cell dropout/apoptosis (Figures 2A and B). In cases with predominantly monocytic inflammation (i.e. few or no neutrophils), the interacinar inflammation may be inconspicuous on routine H&E stains, whereas the monocyte/macrophage infiltrates can be highlighted with the performance of a CD68 immunostain (Figures 2C and D; Refs. 6,14).

- Interacinar capillaritis
  In more severe or advanced forms of acute AMR there is dilatation and congestion of the IAC which allows for easier identification of marginating and intraluminal inflammatory infiltrates (interacinar capillaritis). The latter is similar morphologically and presumably pathogenetically to peritubular capillaritis in renal allografts (56), but in comparison to the renal peritubular capillaries, the pancreatic IAC have a less predictable distribution

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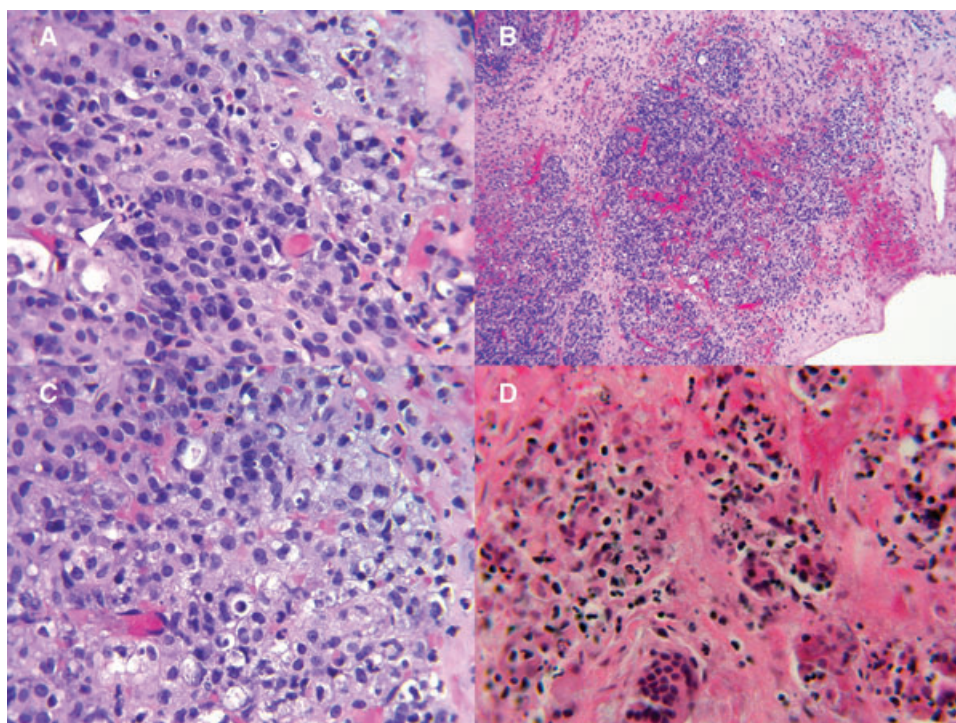
Figure 3: (A and C) Moderate acute AMR characterized by acinar/interacinar inflammation and capillaritis. The acinar cell injury (swelling, vacuolization and cell dropout) appears disproportionate to the extent of the inflammation. Arrowhead (A) marks neutrophilic capillaritis. (B and D) Severe acute AMR represented at low magnification to show marked vascular congestion and confluent areas of hemorrhagic necrosis (B). Exocrine area with multicellular necrosis and mixed interacinar/acinar inflammation (D).

and are relatively sparse. Furthermore in fully developed acute AMR in pancreas allografts, extensive microvascular injury leads to prominent interstitial hemorrhage, edema and multicellular necrosis of interstitial and acinar cells making the identification of interacinar capillaritis more difficult (see later; Figure 3; Ref. 57). Identification of interacinar infiltrates with associated interacinar capillaritis has been found to be strongly associated with C4d positivity and detection of DSA (6,7,12,14). In one study capillaritis was found in >80% of biopsies with focal and diffuse C4d positivity (6).

- Acinar cell and overall tissue injury
  In pancreatic acute AMR, there is increased acinar cell injury manifested with cytoplasmic swelling and vacuolization as well as apoptotic or necrotic cell dropout. The identification of acinar cell injury in an otherwise bland appearing biopsy (Figures 2 and 3) should alert the pathologist to the possibility of subtle interacinar inflammation or capillaritis, and warrants correlation with the C4d staining and DSA studies (6,9,12,14).

In addition to the features described earlier, very severe or advanced forms of acute AMR have morphological features approaching those found in hyperacute rejection. These findings consist of widespread vascular necrosis and thrombosis in small or larger vessels and small or confluent foci of parenchymal necrosis (Figures 1D, 3B and D; Ref. 2).

Acute AMR is graded histologically (mild, moderate or severe) based on the extent of the interacinar infil-

trates/capillaritis and tissue damage, as presented in Table 2.

Chronic active AMR

Chronic exposure to circulating DSA is associated with development of graft fibrosis and graft failure (58). The histological diagnosis of *chronic active AMR* is based on the following triad: (i) features of acute AMR as described in the previous section (also see Table 4; AMR diagnostic components), (ii) absence of features of ACMR and (iii) underlying graft fibrosis (Banff diagnostic category 6). The utilization of this diagnostic category presupposes that the main cause of graft fibrosis is ongoing AMR, and therefore requires that other causes of graft fibrosis/sclerosis are ruled out, such as previous episodes of ACMR. In clinical practice this conclusion would be most accurate when serial biopsies are available for evaluation.

Mixed ACMR and AMR

A generalized increase in interstitial inflammation, both in septa and acini, as well as edema were found to be more common in biopsies with C4d positivity and concurrent circulating DSA (12), which raises the possibility of cases having mixed ACMR and AMR.

Stereotypical cases of isolated AMR or ACMR can be classified by a systematic evaluation of the various features described in Table 3, but it is not unusual for the two processes to coexist in the same biopsy (mixed rejection) and appear with overlapping features. The pathology report should clearly indicate the type of rejection present

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Table 2: Histological grading of acute antibody-mediated rejection (See Table 4*^ for other diagnostic components)

Grade I/Mild acute AMR
Well-preserved architecture, mild monocytic-macrophagic or mixed (monocytic-macrophagic/ neutrophilic) infiltrates with rare acinar cell damage
Grade II/Moderate acute AMR
Overall preservation of the architecture with interacinar monocytic-macrophagic or mixed (monocytic-macrophagic/neutrophilic) infiltrates, capillary dilatation, capillaritis, congestion, multicellular acinar cell dropout and extravasation of red blood cells
Grade III/Severe acute AMR
Architectural disarray, scattered inflammatory infiltrates in a background of interstitial hemorrhage, multifocal and confluent parenchymal necrosis, arterial and venous wall necrosis and thrombosis

(AMR, ACMR or mixed), estimate the degree of activity (mild, moderate or severe) of each process and indicate the extent of chronicity/graft fibrosis (stage).

Specific Considerations on the Updated Banff Schema for Grading Pancreas Allograft Rejection

1. It is now recognized that one of the main features of acute AMR in the pancreas is the presence of prominent acinar cell injury ranging from spotty cell necrosis/apoptosis to confluent necrosis. Accordingly it is now stated in the schema that Grade II and III/Moderate and Severe ACMR, which can also present with prominent acinar cell injury/necrosis, require differentiation from acute AMR (Table 4).

2. Diagnosis of acute AMR is based on a combination of diagnostic components as listed in Table 4. Criteria for the histological grading of acute AMR (mild, moderate or severe) are provided in a separate table (Table 2).

3. Chronic active AMR is based on the combination of acute AMR and graft sclerosis-fibrosis (Categories 4 and 6), in the absence of ACMR (Category 3). A conclusive diagnosis of *chronic active AMR* requires C4d positivity, interacinar inflammation/capillaritis/acinar damage and circulating DSA in addition to graft sclerosis-fibrosis. If only two of the AMR diagnostic elements are present, a diagnosis of *suspicious for chronic active AMR* can be rendered.

4. Chronic allograft arteriopathy was initially considered to be an expression of T-cell-mediated allograft rejection (45), but recent studies have shown that acute and chronic arterial lesions can be also associated with DSA and AMR (59–61). Accordingly, this lesion is now listed as a separate morphological category (independent from ACMR and AMR). Recognition of chronic allograft arteriopathy in biopsy samples is clinically important because it indicates ongoing (chronic) alloimmune injury and for its association with late graft thrombosis (2).

5. A separate category has been created for lesions specifically involving the endocrine islets. The main purpose of this category is the recognition of *recurrent autoimmune diabetes mellitus*, characterized by insulitis and/or selective ß cell loss (62,63). In addition, islet deposition of Amylin (also known as islet amyloid polypeptide (IAPP)) appearing as amorphous Congo red positive material is placed in this category. Amylin, a protein normally cosecreted with insulin by ß cells, accumulates in the pancreatic islets under abnormal circumstances in particular hyperglycemic states (i.e. Type 2 diabetes mellitus), pancreatitis and possible allograft rejection (64,65). Deposition of amylin in otherwise normal islets of pancreas allografts is usually associated with loss of glycemic control (hyperglycemia). Acute calcineurin inhibitor toxicity (9) is also included in this category.

The impact of AMR in pancreatic islets remains unclear. Whereas hyperglycemia was documented in early reports of AMR (13,58), this was a rare indication for biopsy in subsequent larger studies (6,12,15). C4d staining in islet capillary endothelium was found in

Table 3: Predominance of histological features in stereotypical ACMR and AMR

	ACMR	AMR
Septal infiltrates	+++	− to +
Eosinophils	+ to +++	− to +
Neutrophils	− to ++	+/− to +++
T-Lymphocytes	++ to +++	+/− to +
Macrophages	++	++++
Venulitis	++	−
Ductitis	++	−
Acinar cell injury	+/− to ++	+++
Acinar inflammation	− to +++	+ to +++
Acinitis (mononuclear infiltrates within the basement membrane of individual acini)	+ to +++	− to+/−
Interacinar capillaritis	− to +/−	+ to +++
Intimal arteritis	+	+
Necrotizing vasculitis/thrombosis	− to +	+++
Confluent hemorrhagic necrosis	− to ++	− to ++++
Active transplant arteriopathy	+	+

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Table 4: Banff pancreas allograft rejection grading schema—update diagnostic categories#

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1. Normal. Absent inflammation *or* inactive septal, mononuclear inflammation not involving ducts, veins, arteries or acini. There is no graft sclerosis. The fibrous component is limited to normal septa and its amount is proportional to the size of the enclosed structures (ducts and vessels). The acinar parenchyma shows no signs of atrophy or injury.

2 Indeterminate. Septal inflammation that appears active but the overall features do not fulfill the criteria for mild cell-mediated acute rejection

3. Acute T-cell-mediated rejection[+]
- Grade I/Mild acute T-cell-mediated rejection

Active septal inflammation (activated, blastic lymphocytes and ±eosinophils) involving septal structures: Venulitis (subendothelial accumulation of inflammatory cells and endothelial damage in septal veins, ductitis (epithelial inflammation and damage of ducts)

and/or

Focal acinar inflammation. No more than two inflammatory foci[^]per lobule with absent or minimal acinar cell injury
- Grade II / Moderate acute T-cell-mediated rejection (requires differentiation from AMR)

Multifocal (but not confluent or diffuse) acinar inflammation ($\geq$3 foci[^]per lobule) with spotty (individual) acinar cell injury and dropout

and/or

Mild intimal arteritis (with minimal, <25% luminal compromise)
- Grade III / Severe acute T-cell-mediated rejection (requires differentiation from AMR)

Diffuse (widespread, extensive) acinar inflammation with focal or diffuse multicellular/confluent acinar cell necrosis

and/or

Moderate or severe intimal arteritis, >25% luminal compromise.

and/or

Transmural inflammation—Necrotizing arteritis.

4. Antibody-mediated rejection (AMR*, See diagnostic components below*)

*Confirmed circulating donor-specific antibody (DSA)

*Morphological evidence of tissue injury (interacinar inflammation/capillaritis, acinar cell damage swelling/necrosis/apoptosis/dropout, vasculitis, thrombosis)

*C4d positivity in interacinar capillaries (IAC, $\geq$5% of acinar lobular surface)

Acute AMR 3 of 3 diagnostic components*[^]

Consistent with acute AMR 2 of 3 diagnostic components*[^]

Requires exclusion of AMR 1 of 3 diagnostic components*

See separate table for histological grading of acute AMR[^]

Chronic active antibody-mediated rejection: Combined features of categories 4* and 6 in the absence of features of category 3

5. Chronic allograft arteriopathy. Arterial intimal fibrosis with mononuclear cell infiltration in fibrosis.

6. Chronic allograft rejection/graft fibrosis
- Stage I (mild graft fibrosis)

Expansion of fibrous septa; the fibrosis occupies less than 30% of the core surface but the acinar lobules have eroded, irregular contours. The central lobular areas are normal.
- Stage II (moderate graft fibrosis)

The fibrosis occupies 30–60% of the core surface. The exocrine atrophy affects the majority of the lobules in their periphery (irregular contours) and in their central areas (thin fibrous strands criss-cross between individual acini).
- Stage III (severe graft fibrosis)

The fibrotic areas predominate and occupy more than 60% of the core surface with only isolated areas of residual acinar tissue and/or islets present.

7. Islet pathology

Recurrence of autoimmune DM (insulitis and/or selective ß cell loss)

Islet amyloid (amylin) deposition

8. Other histologic diagnosis. Pathologic changes not considered to be due acute and/or chronic rejection. For example, CMV pancreatitis, PTLD, etc.

#Categories 2 to 8 may be diagnosed concurrently and should be listed in the diagnosis in the order of their clinicopathological significance.
+Histological features of stereotypical ACMR and AMR, see Table 3.
^Histological grading of acute AMR, see Table 2.
See Ref. 9 for morphological definition of lesions of cell-mediated rejection and for list of other histological diagnosis.

approximately 20% of samples from patients with DSA but this finding did not correlate with hyperglycemia (6). In severe necrotizing AMR (as well as severe ACMR) hyperglycemia typically develops, correlating with the extent of parenchymal necrosis (2,9).

6. The proposed schema for diagnosis of AMR in pancreas allografts follows the same approach as the Banff 09 update for diagnosis of acute AMR in kidney allografts (66). Both schemas rely on the combination of C4d positivity, presence of circulating DSA and

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evidence of associated tissue injury for a diagnosis of *acute AMR*. In both schemas, a diagnosis of *suspicious for AMR* is rendered in the absence of any one of these diagnostic components (66).

Discussion

AMR is a complex, dynamic process with protean clinicopathological manifestations that range from cataclysmic graft loss to various forms of allograft inflammation associated with protracted graft sclerosis-fibrosis (67). From a practical point of view, severe untreatable acute AMR can be easily diagnosed on the basis of morphological criteria alone, but a systematic approach and high degree of suspicion are necessary in order to recognize the milder or more indolent and potentially treatable forms of the disease (9,17,45). Biopsy evaluation remains the gold standard for the diagnosis of allograft rejection, as clinical parameters lack specificity and cannot discriminate between ACMR and AMR which require different therapeutic approaches (15).

Detailed histological examination, immunostaining for C4d and serological search for circulating DSA currently represent the cornerstones for the diagnosis of AMR, although significant questions remain with respect to determining the most adequate tools and thresholds for this diagnosis (16,44,67). In particular, fluctuating levels of DSA (and C4d staining) as well as technical limitations for the identification of DSA have been problematic (36–38). However, other promising tools are not yet available for routine diagnosis (3,16,68).

The schema proposed here relies on the combination of the currently available tools (DSA, C4d and histological findings), based on their perceived strengths and limitations, and also their complementary value (12,45; see Table 4). A diagnosis of "suspicious for AMR" increases the sensitivity of the schema by addressing the not too unlikely clinical situation in which a complete constellation of elements is not identified (i.e. only two of the three elements are present). On the other hand, the presence of C4d positivity, DSA or tissue injury in isolation from the other elements is not considered sufficient to warrant clinical intervention for AMR but should prompt thorough clinicopathological correlation and close follow-up.

Currently there is no adequate treatment for either acute or chronic AMR. This process is currently one of the most challenging problems in solid organ transplantation (10,16,21). Interventions in pancreas AMR have followed the approach used in other organs, mainly consisting of rabbit antithymocyte globulin, intravenous immune globulin (IVIG) and plasmapheresis with or without rituximab. Less well-established treatments include the addition of bortezomib and/or eculizumab, but these therapies need to be evaluated in formal clinical trials (69).

The goal of this Banff working proposal is to provide uniform diagnostic criteria that can be applied both for diagnostic and investigational purposes in pancreas transplantation. Morphological classifications such as this one have inherent limitations related to intra- and interobserver reproducibility (70). Studies are being currently undertaken to evaluate reproducibility issues in this specific context.

Future Direction

In the field of pancreas transplantation there are multiple areas of investigation that require attention, some of which are listed below.

Clinical studies

- Need for better understanding of the pathogenetic mechanisms in AMR including better characterization of the role of presensitization, role of surveillance DSA/PRA, determination of meaningful clinical cut-offs for DSA levels (e.g. highest levels vs. broader sensitization), potential role of circulating non-HLA antidonor antibodies and autoantibodies (including antiislet antibodies and other autoantibodies to SMA or collagen), impact of AMR on the exocrine versus endocrine components and relationship between alloimmunity and autoimmune recurrence.
- Single-center and multicenter studies to determine the utility of protocol biopsies enabling identification and determination of the significance of subclinical rejections, early identification of AMR versus ACMR and refinement of clinicopathological correlations.

Pathological studies

- Histopathological characterization of the inflammatory infiltrates in AMR and ACMR (e.g. immunohistochemical application of lymphoid markers) to improve diagnostic yield and increase data accumulation of pathophysiologic significance.
- Further refinement of the morphological characterization of microvascular and endothelial injury and study of the pathogenesis of chronic rejection/graft sclerosis.

Molecular studies

- Application to pancreas transplantation of the currently available tools in gene profiling, gene transcription and proteomics to improve understanding of the pathogenetic mechanisms of allograft rejection (ACMR, AMR and mixed rejection) and other processes leading to graft failure.
- Exploration of the potential use of limited microarray analysis or multiplex polymerase chain reaction (PCR) for obtaining diagnostic and pathophysiologic data (e.g. PCR for Th1, Th2, Th17 cytokines or cells).

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Individual efforts in these areas will advance understanding of basic mechanisms and enhance clinical management of pancreas allografts. This review and update of the Banff grading schema for AMR will hopefully provide standardization, improve diagnosis and understanding and help elucidate mechanisms of graft failure as well as target interventions for improving long-term outcomes.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose, as described by the *American Journal of Transplantation*.

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A0234

A SCHEMA FOR HISTOLOGIC GRADING OF SMALL INTESTINE ALLOGRAFT ACUTE REJECTION

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Background. Histologic evaluation of small bowel allograft biopsies is important for the diagnosis of acute rejection. However, a standard histologic schema to grade the severity of intestinal acute rejection is not currently available. The primary goal of this study was to develop a histologic grading system for the diagnosis of small bowel allograft acute rejection.

Methods. We evaluated 3268 small bowel allograft biopsies obtained from adult patients who underwent small bowel transplantation at the University of Pittsburgh Medical Center between 1990 and 1999. A histologic grading system was proposed and validated by retrospective correlation with clinical outcomes.

Results. Among the 3268 biopsies, 180 acute rejection episodes were diagnosed (88 indeterminate, 74 mild, 14 moderate, and 4 severe). All four histologically diagnosed, severe acute rejection episodes resulted in graft failure before resolution, despite aggressive immunosuppressive therapy. Four of the 14 moderate acute rejection episodes were associated with unfavorable clinical outcomes. In contrast, the 74 mild and 88 indeterminate acute rejection episodes were not associated with unfavorable clinical outcomes. Statistical analysis for trend revealed that grades indicating more severe acute rejection episodes were associated with a greater probability of unfavorable outcomes ($P<0.01$). In addition, there was good overall agreement among different pathologists regarding the diagnosis of acute rejection using the proposed schema, suggesting that this system is practical.

Conclusions. This study provides a reliable predictive schema for assessment of the severity of human small bowel acute rejection.

Small bowel transplantation is being increasingly performed to treat patients with irreversible intestinal failure or short-bowel syndrome (*1–7*). Acute cellular rejection (ACR) is the major cause of intestinal graft failure after transplantation (*8*). If not treated early, intestinal ACR can rapidly increase in severity and cause graft failure and death. In fact, despite aggressive immunosuppressive therapy, most patients with histologically diagnosed severe acute rejection experience progression to graft loss or death. Therefore, accurate diagnosis and treatment of acute rejection are critical for posttransplant patient care.

The diagnosis of intestinal ACR requires close correlation of clinical, endoscopic, and pathologic findings. The clinical symptoms of intestinal ACR include fever, nausea, vomiting, increased stomal output, abdominal pain, and distension. In severe cases, acute rejection may manifest as septic shock, with metabolic acidosis, hypotension, and adult respiratory distress syndrome, which likely results from loss of mucosal integrity and bacterial translocation across the intestinal wall. The endoscopic appearances of intestinal ACR range from edema and hyperemia in mild cases to granularity, loss of the fine mucosal vascular pattern, diminished peristalsis, and mucosal ulceration in more severe cases. The final diagnosis depends on histologic analysis of endoscopy-guided mucosal biopsy specimens. The major histopathologic changes of intestinal ACR were documented in previous studies (*8, 9*) and include varying degrees of (1) infiltration by a mixed but primarily mononuclear inflammatory population, including blastic or activated lymphocytes; (2) crypt injury (characterized by cytoplasmic basophilia, nuclear enlargement and hyperchromasia, decreased cell height, mucin depletion, and loss of Paneth's cells); (3) increases in the number of crypt apoptotic bodies; and (4) distortion of villous and crypt architecture.

The treatment options for intestinal ACR depend on its severity, which is assessed by histologic grading of the rejection with clinical and endoscopic correlation. For example, whereas relatively mild acute rejection usually requires an increase in basal immunosuppressive drug treatment with close clinical observation, more aggressive immunosuppressive therapy should be initiated for moderate or severe episodes of acute rejection. Therefore, accurate grading of acute rejection is extremely important for successful patient treatment. Histopathologic grading of acute rejection has not yet been addressed in detail, however, and no standard criteria are available for assessment of the grade of intestinal ACR. The major goal of this study was to develop a reliable, practical histologic grading system for pathologic evaluation of human intestinal ACR. On the basis of results from animal intestinal transplantation studies (*10–12*) and clinical experience in evaluating thousands of small bowel allograft biopsies in our institution (*8*), we proposed a pathologic grading system for the diagnosis of intestinal ACR. This system was used to retrospectively evaluate 3,268 small bowel allograft biopsies from 52 adult patients who underwent intestinal transplantation between 1990 and 1999 at the Thomas E. Starzl Transplant Institute, University of Pittsburgh Medical Center. The histologic grades determined were then correlated with clinical events, including immunosuppressive therapy and graft and patient outcomes. Our results indicate that the proposed grading system is accurate in the diagnosis

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of intestinal ACR and is practical for routine histologic evaluation of intestinal allograft specimens.

MATERIALS AND METHODS

Patient Population

During the 9-year period between May 1990 and June 1999, 52 adult patients (26 male and 26 female patients; age range, 19–58 years) underwent orthotopic intestinal transplantation at the University of Pittsburgh Medical Center. The patient demographic characteristics, types of procedures, and causes of intestinal failure are summarized in Table 1. Baseline immunosuppressive therapy consisted of administration of tacrolimus and corticosteroids (*1*). Details of graft procurement, surgical procedures, tacrolimus-based immunosuppressive therapy, and patient treatment were reported previously (*1*, *2*). Surveillance allograft endoscopy was generally performed once or twice per week for the first 3 months and as clinically indicated thereafter. Multiple random, endoscopy-guided biopsies were routinely obtained from the small intestinal allograft (most often from the ileum) for histologic evaluation. Each biopsy specimen consisted of one to five separate mucosal fragments (median of three). The relevant clinical features and course of each patient were retrieved from our computerized database, and missing data were obtained in reviews of patient flow sheets and medical records. Complete follow-up data were available through the completion of the study (June 30, 1999).

TABLE 1. Demographic summary of patients with small intestine transplants

No. of patients	52
Gender (male:female)	26:26
Age range (yr)	19–58
Types of grafts (55 grafts, with 3 cases of retransplantation)	
Isolated intestine	29 (including colon in 8)
Small bowel/liver	16
Small bowel/pancreas	1
Multivisceral	9 (including colon in 4)
Causes of intestinal failure (no. of cases)	
Vascular thrombosis	17
Crohn's disease	12
Abdominal trauma	7
Mesenteric fibromatosis	5
Volvulus	3
Surgical adhesions	2
Radiation-induced enteritis	2
Familial polyposis	2
Pseudo-obstruction	1
Metastatic gastrinoma	1

Histologic Evaluations

All pathologic specimens from the 55 intestinal allografts were reviewed, including 3268 small intestinal mucosal biopsies. The histologic specimens were routinely fixed in formalin and embedded in paraffin, from which 2 to 18 hematoxylin-eosin–stained sections were obtained, from two or more levels in the blocks. Samples were obtained from deeper levels as indicated. For each specimen, the major histologic features, including architectural distortion (villous blunting, as determined in the best-oriented sections), crypt epithelial injury (characterized by cytoplasmic basophilia, nuclear enlargement and hyperchromasia, decreased cell height, and mucin depletion), inflammatory infiltration of the lamina propria and the constituent cell types, presence and cell type of crypt intraepithelial infiltration (cryptitis), lamina propria fibrosis, granulation tissue, and luminal fibrinopurulent inflammatory exudation (pseudomembrane), were semiquantitatively assessed. In addition, the specimens were carefully examined for viral infections, luminal organisms, and submucosal abnormalities. Apoptotic bodies within the crypt epithelium were identified and quantified. Apoptotic bodies were defined as rounded vacuoles containing fragments of karyorrhectic nuclear debris and cytoplasm and were distinguished from small isolated fragments of nuclear chromatin and intraepithelial neutrophils and eosinophils. These bodies were counted by scanning the specimen at medium power, to identify areas of greatest concentration, and then tallying the total numbers in 10 consecutive crypts (regardless of crypt orientation), including more than one level if necessary.

Slides from all biopsy specimens were reviewed at least twice by at least two pathologists. Histologic features relevant to acute rejection were compiled during the initial review; and a list of biopsy features of rejection was recorded by the second pathologist. Ambiguous or difficult cases were further reviewed using a multihead microscope by three or four pathologists. Attention was focused on changes related to rejection (see later discussion).

Histologic Criteria for Grading of Acute Cellular Rejection

The proposed histologic grading system for small bowel allograft biopsies is based on previous animal studies (*10–12*) and our clinical experience in the evaluation of thousands of small bowel allograft biopsy specimens (*8*). The histologic criteria for grading intestinal ACR are summarized (Table 2).

Indeterminate for acute rejection. Indeterminate for acute rejection is defined by the variable presence of the three main features of acute rejection (infiltration by a mixed but primarily mononuclear inflammatory population, including blastic or activated lymphocytes; crypt injury; and increased numbers of crypt apoptotic bodies), which are usually focal and do not meet the criteria for mild acute rejection. The inflammatory infiltrate is usually minimal and localized. Although the mucosa is intact, crypt epithelial injury is often present. There is a variable increase in crypt epithelial apoptosis but usually with less than 6 apoptotic bodies per 10 crypts (Fig. 1). *Indeterminate for acute rejection* should be used only when the biopsy demonstrates

TABLE 2. Histologic criteria for grading of small bowel allograft acute rejection[a]

Grade	Major Histologic Findings
Indeterminate for ACR	Minimal localized inflammatory infiltrate, minimal crypt epithelial injury, increased crypt epithelial apoptosis (usually with <6 apoptotic bodies/10 crypts), no to minimal architectural distortion, no mucosal ulceration, changes insufficient for the diagnosis of mild acute rejection
Mild ACR	Mild localized inflammatory infiltrate with activated lymphocytes, mild crypt epithelial injury, increased crypt epithelial apoptosis (usually with >6 apoptotic bodies/10 crypts), mild architectural distortion, no mucosal ulceration
Moderate ACR	Widely dispersed inflammatory infiltrate in lamina propria, diffuse crypt epithelial injury, increased crypt apoptosis with focal confluent apoptosis, more prominent architectural distortion; possible mild to moderate intimal arteritis; no mucosal ulceration
Severe ACR	Features of moderate ACR plus mucosal ulceration; possible severe intimal arteritis or transmural arteritis may be seen

[a] ACR, acute cellular rejection.

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FIGURE 1. Indeterminate for acute rejection. The lamina propria is infiltrated by a heterogeneous population of mononuclear cells composed of blastic and small lymphocytes, plasma cells, and plasmacytoid lymphocytes. There is focal minimal crypt damage and apoptotic bodies (*arrow*) (hematoxylin-eosin; magnification: ×200 in A, ×400 in B). The apoptotic body count is usually less than 6 apoptotic bodies per 10 crypts.
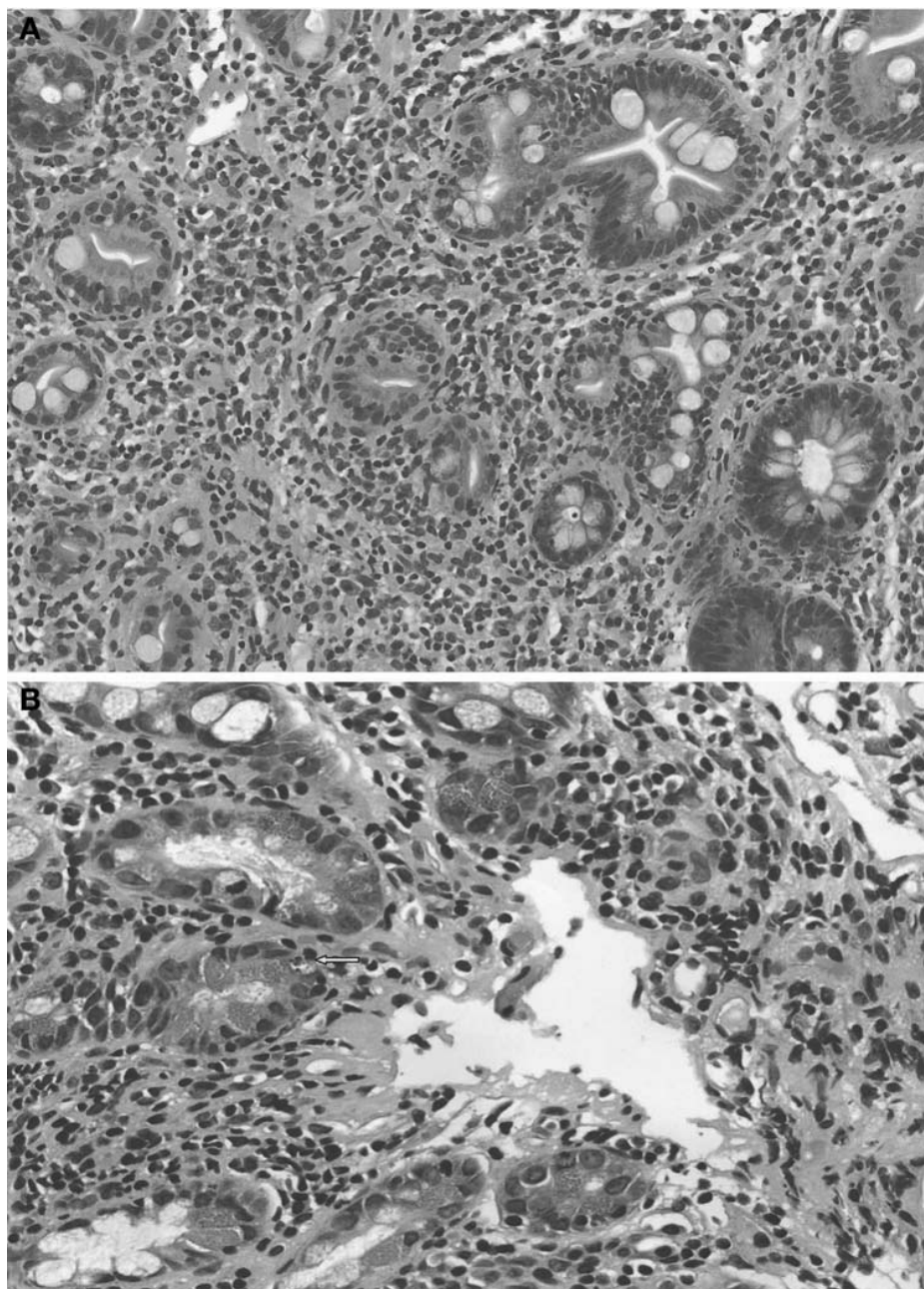
features of acute rejection with degrees of inflammation, epithelial injury, and apoptosis that are lesser than those for mild acute rejection; it should not be applied to nonrejection processes when the diagnosis is not clear.

Mild acute rejection. Mild acute rejection is characterized by a generally mild and localized inflammatory infiltrate, which tends to be concentrated around small venules in the lamina propria. The mucosa is intact, but the crypt epithelium displays evidence of injury, including mucin depletion, cytoplasmic basophilia, decreased cell height, nuclear enlargement and hyperchromasia, and inflammatory infiltration. Crypt epithelial apoptosis is increased, usually with more than 6 apoptotic bodies per 10 crypts. If sampled in the biopsy specimen, preexisting lymphoid aggregates (Peyer's patches) demonstrate an intense accumulation of activated lymphocytes. The villi are variably shortened, and the architectural features may be slightly distorted because of expansion of the lamina propria by inflammatory infiltration (Fig. 2).

Moderate acute rejection. In *moderate acute rejection*, the inflammatory infiltrate is widely dispersed within the lamina propria. Crypt damage is distributed more diffusely than in mild acute rejection, and the villi tend to exhibit a greater degree of flattening. The number of apoptotic bodies is greater than in mild acute rejection, usually with focal "confluent apoptosis." Mild to moderate intimal arteritis may be observed. The mucosa remains intact without ulceration, although focal superficial erosions can be present (Fig. 3).

Severe acute rejection. Severe acute rejection is distinguished by a marked degree of crypt damage and mucosal ulceration. As a consequence of the mucosal destruction, luminal contents gain access to the submucosa, prompting a neutrophil-rich infiltrate and an overlying fibropurulent (pseudomembranous) exudate, with widespread

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TRANSPLANTATION
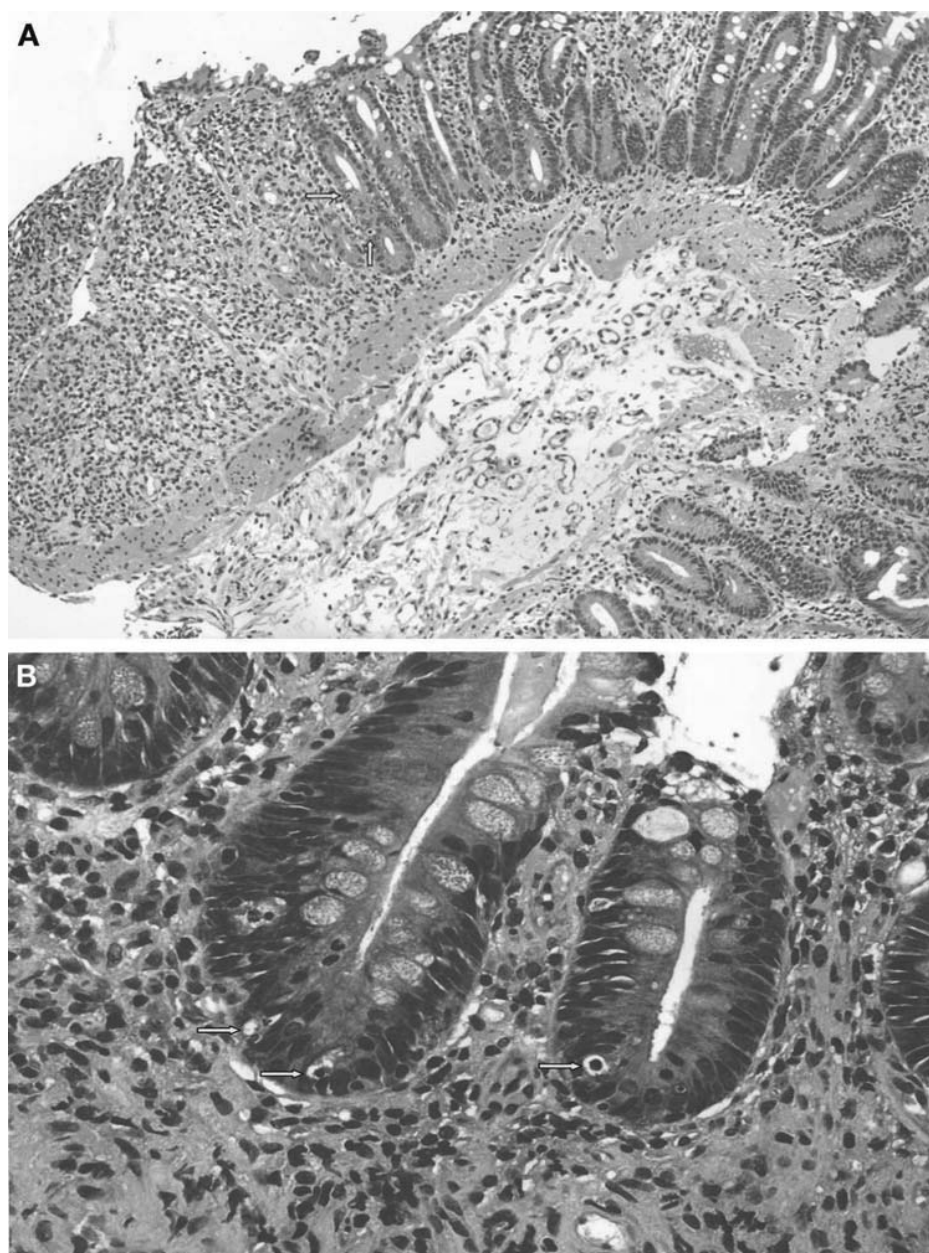
FIGURE 2. Mild acute rejection. (A) The villi are shortened and the architectural features are distorted because of expansion of the lamina propria by the heterogeneous mononuclear cell infiltrate (*left*). The crypts exhibit features of epithelial injury and scattered apoptotic bodies (*arrows*) (hematoxylin-eosin; magnification ×100). (B) Lamina propria mononuclear inflammation, crypt epithelial injury, and apoptotic bodies (*arrows*) (clear spaces with fragmented nuclear debris) (hematoxylin-eosin; magnification ×400). The apoptotic body count in mild acute rejection is usually more than six apoptotic bodies per 10 crypts.

mucosal sloughing as the final result. The adjacent viable epithelium usually exhibits rejection-associated changes, such as crypt epithelial damage and abundant apoptosis (Fig. 4). Severe intimal arteritis or transmural arteritis may be observed.

Prognostic Use of the Grading System

To evaluate the ability of the proposed acute rejection grading system to predict an unfavorable outcome, the histologic diagnoses of acute rejection episodes were retrospectively correlated with the clinical outcomes and treatments. A biopsy was defined as representing an acute rejection episode if the biopsy specimen was the first one to be histologically diagnosed as acute rejection. A new rejection episode was defined by newly developed clinical symptoms and documentation of new histologic features of ACR with at least one normal mucosal biopsy between the rejection episodes. For endpoint analysis, patients were divided into groups with favorable or unfavorable outcomes. Objective unfavorable outcomes were defined by

the presence of any one of the following: (1) the rejection resulted in graft failure (death or retransplantation) before resolution; (2) OKT3 or antithymoglobulin was required for the treatment of acute rejection; or (3) complete resolution of the episode failed to occur within 21 days.

Reliability of the Grading System

Sixty-five posttransplantation small bowel biopsy specimens were randomly selected and reviewed by four pathologists. Before reviewing the slides, the pathologists agreed on the histologic grading criteria. Each participating pathologist rendered a final histologic diagnosis on the basis of the standard criteria.

Statistical Analyses

The ability of the grading system to predict an unfavorable outcome was assessed with the chi-square test for trend, using the definitions for unfavorable outcomes. The agreement among pathol-

FIGURE 3. Moderate acute rejection. Crypt damage and apoptosis are distributed more diffusely than in mild acute rejection. The number of apoptotic bodies is greater than in mild acute rejection, with focal confluent apoptosis (*arrows*). The mucosa is usually intact, without ulceration (hematoxylin-eosin; magnification ×200).
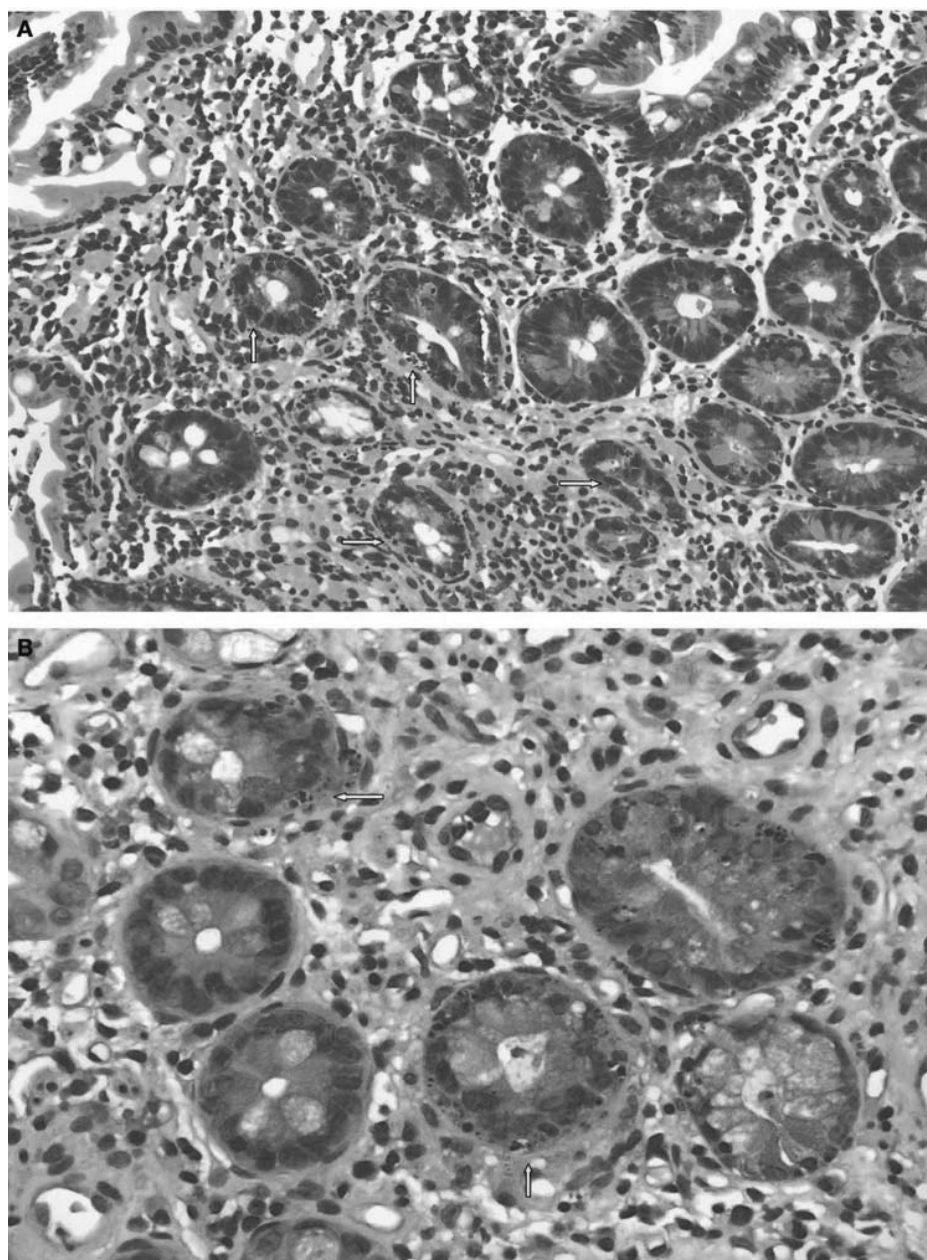
ogists regarding the histologic diagnosis of ACR was analyzed with multirater kappa analysis.

RESULTS

Histologic Diagnosis of Acute Cellular Rejection

The histologic diagnosis and grading of ACR were performed after careful evaluation of 3268 mucosal biopsies from 55 small intestinal allografts. The initial histologic diagnosis for each biopsy specimen was established by the primary pathologist during the daily signed-out process; each of the biopsies was reevaluated by a separate pathologist (T.W.), and detailed histologic features were recorded. If an ambiguity regarding any histologic feature or a disagreement in diagnoses existed, then the slides were further reviewed under a multihead microscope with two or more additional pathologists, and the consensus opinion was recorded. A biopsy was defined as representing an acute rejection episode if the biopsy specimen was the first one to be histologically diagnosed as acute rejection. A new rejection episode was defined on the basis of newly developed clinical symptoms and documentation of new histologic features of ACR, with at least one normal mucosal biopsy between the rejection episodes. On the basis of the aforementioned criteria, 180 episodes of ACR were histologically diagnosed, among which were 88 (49%) episodes of indeterminate for ACR, 74 (41%) episodes of mild ACR, 14 (8%) episodes of moderate ACR, and 4 (2%) episodes of severe ACR. Among the 180 episodes of histologically diagnosed ACR (including indeterminate for ACR), 85 (47%) episodes occurred within the first 2 months after transplantation, 46 (26%) episodes occurred 2 to 12 months after transplantation, 24 (13%) episodes occurred 1 to 2
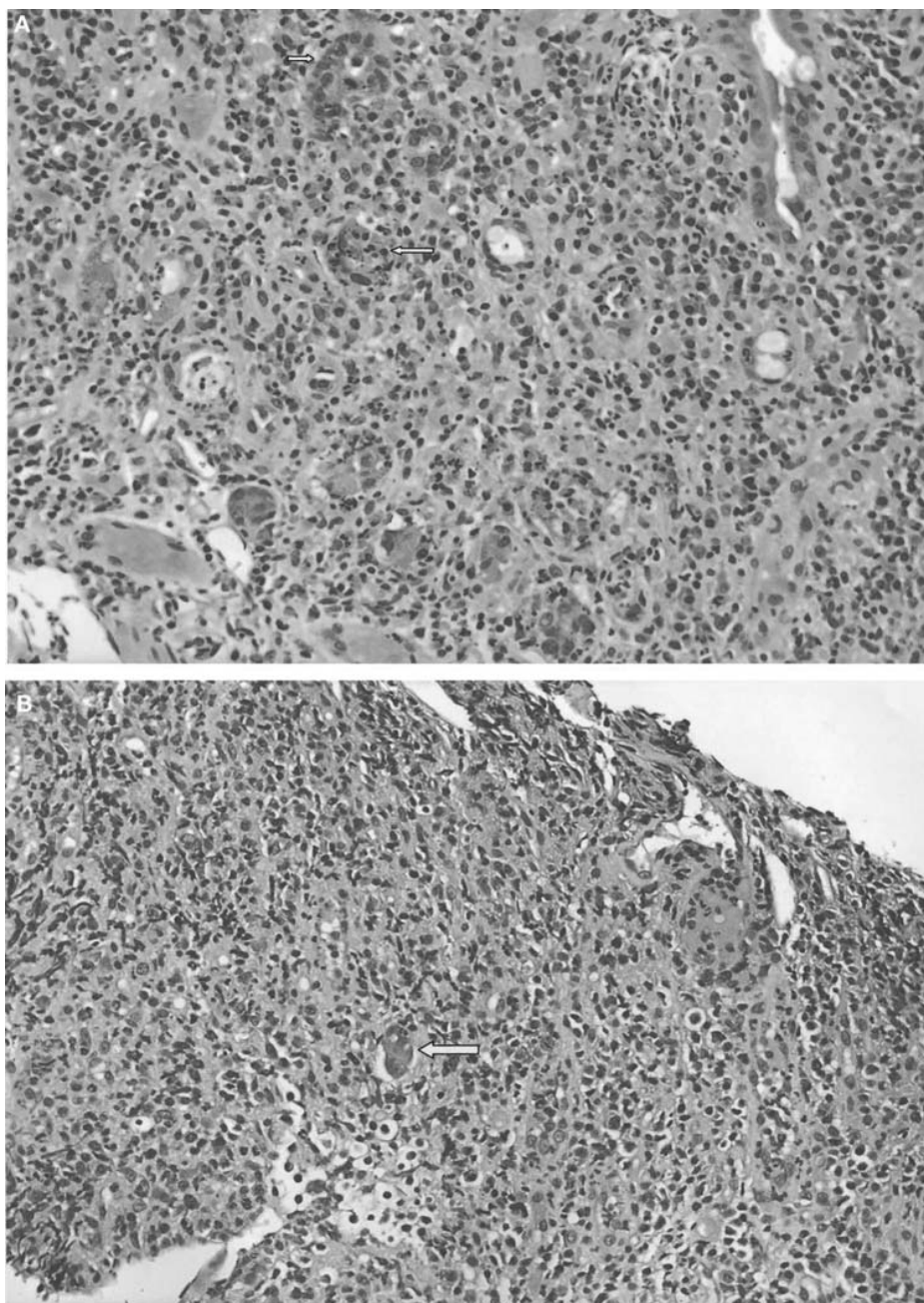
FIGURE 4. Severe acute rejection. There is extensive mucosal destruction, with loss of crypts, mucosal ulceration, and mixed lymphoplasmacytic, eosinophilic, and neutrophilic infiltration. The residual crypts, if present, often exhibit marked epithelial injury and apoptosis (*arrows*) (hematoxylin-eosin; magnification ×200).

years after transplantation, and 25 (14%) episodes occurred more than 2 years after transplantation.

The same histologic grading criteria were used for all biopsies in this study, including biopsies obtained from patients with clinical symptoms and protocol biopsies. The clinical presentations associated with ACR included abdominal pain, nausea, vomiting, diarrhea, fever, and abdominal distention. These symptoms lacked specificity, however, and varied depending on the severity of rejection and the presence of other pathologic conditions, such as acute enteritis, cytomegalovirus (CMV) infection, intestinal obstruction, systemic infection, or posttransplant lymphoproliferative disorder (PTLD). All of the patients with histologic diagnoses of moderate or severe ACR exhibited

clinical symptoms, and approximately 95% of the patients with histologic diagnoses of mild or indeterminate acute rejection exhibited symptoms. The remaining 5% of patients with mild or indeterminate acute rejection exhibited no symptoms at the time of the biopsies, and the diagnoses were established with protocol biopsies. Most of the biopsies without histologic evidence of acute rejection demonstrated either normal mucosa or mild nonspecific enteritis; some showed reparative mucosa, CMV infection, Epstein-Barr virus (EBV) infection, or PTLD.

Prognostic Ability of the Grading System

We then wished to analyze the association between acute rejection grades and unfavorable outcomes. For this purpose,

the patients were divided into those with favorable outcomes and those with unfavorable outcomes, according to the aforementioned criteria, and the ability of the grading system to predict an unfavorable outcome was assessed with the chi-square test for trend. The results demonstrated that a grade indicating a more severe rejection episode was associated with a greater probability of an unfavorable outcome ($P<0.01$). In fact, all four of the histologically diagnosed severe acute rejection episodes resulted in graft failure before resolution, despite treatment with OKT3. Of those four grafts, three were removed because of uncontrolled ACR and one patient died as a result of ACR with the graft in place. Of the 14 episodes of moderate acute rejection, 2 episodes required OKT3 treatment and 2 episodes failed to resolve within 21 days with immunosuppressive therapy (other than OKT3). The outcome of one moderate ACR episode could not be determined because of graft removal secondary to chronic rejection, before the resolution of ACR. The remaining nine episodes of histologically diagnosed moderate ACR were not associated with unfavorable outcomes. The outcomes were difficult to assess for 3 of the 74 episodes of mild ACR, because of graft removal in 2 cases (because of chronic rejection and opportunistic infection) and patient death in 1 case (resulting from opportunistic infection) before resolution of the ACR episodes. The remaining 71 mild ACR episodes were not associated with unfavorable outcomes. The 88 indeterminate ACR episodes all resolved within 21 days (spontaneous resolution without treatment, resolution after increased immunosuppressive therapy, or progression to mild ACR that latter resolved with treatment) and were not associated with unfavorable outcomes.

Reliability of the Grading System

A consensus diagnosis was reached by all of the participating pathologists in 60 of the 65 cases (92%), including 4 cases of severe acute rejection, 9 cases of moderate acute rejection, 10 cases of mild acute rejection, 13 cases of indeterminate for ACR, and 24 cases of no acute rejection. Of the five cases for which a uniform diagnosis could not be established, two cases were interpreted as either mild ACR or indeterminate for ACR and three cases were interpreted as either indeterminate or no ACR. There was no disagreement regarding the diagnosis of moderate or severe acute rejection. Multirater kappa analysis demonstrated that there was excellent overall agreement among pathologists regarding the diagnosis and grading of small bowel acute rejection with this grading schema ($P<0.01$). Good intraobserver agreement was noted when the slides were reviewed in a blinded manner by the same pathologist on two separate occasions (with an interval of approximately 6 months).

DISCUSSION

The primary goal of this study was to develop a histologic grading system for the diagnosis of small bowel allograft ACR. To achieve this, we evaluated 3,268 small bowel allograft biopsies obtained from adult patients who underwent small bowel transplantation at our institute during the past decade. On the basis of previously documented major histologic parameters for small bowel allograft acute rejection, the severity of acute rejection was graded as indeterminate, mild, moderate, or severe. This grading system was validated by retrospective correlation with clinical outcomes; more severe rejection episodes were associated with a greater probability of unfavorable clinical outcomes. The excellent overall agreement among different pathologists regarding the histologic diagnosis of acute rejection using the proposed criteria suggests that this system is reliable for the routine pathologic evaluation of small bowel allograft acute rejection. To our knowledge, the criteria in this study represent the first schema for assessment of acute rejection severity in human small bowel allografts.

Several pitfalls in the histologic evaluation of small bowel mucosal biopsies are worth mentioning. We observed that four histologic features are particularly useful for the routine pathologic diagnosis of small bowel allograft ACR, including architectural distortion, crypt apoptosis, crypt epithelial injury, and activated lymphocytic inflammatory infiltration in the lamina propria. These are relatively easily identifiable features that can be reliably quantitatively or semiquantitatively assessed, with a high degree of reproducibility among different pathologists. Because artery sampling is extremely rare in intestinal mucosal biopsies, arteritis has limited diagnostic value in the evaluation of mucosal biopsy specimens, although its presence invariably indicates moderate or severe acute rejection. In this study, arteritis was identified in only 2 of the 3,268 mucosal biopsies. If biopsies are obtained from isolated ulcers or necrotic regions, then an exact histologic diagnosis of acute rejection may be difficult to establish. In such circumstances, careful clinical and endoscopic correlation is particularly important and repeated biopsies from nonulcerated regions are often required.

The quality of the infiltrate (activated lymphocytes mixed with some eosinophils and neutrophils in ACR, compared with nonactivated lymphocytes in nonspecific enteritis) is important in the differentiation of ACR from other conditions. The intensity of the infiltration is generally correlated with the severity of ACR (mild infiltration in mild ACR and intense infiltration in severe ACR). In our experience, the area of infiltration is a less-reliable marker, because the infiltration in low-grade ACR can be diffuse (although less intense). Although eosinophils are frequently observed in intestinal mucosa, significantly increased levels of eosinophils with coexistent activated lymphocytes and crypt apoptosis suggest acute rejection. Peyer's patches are commonly sampled in mucosal biopsies, especially from the ileum. Although localized Peyer's patches without significant lymphoid activation do not indicate acute rejection, Peyer's patches with lymphoid activation (characterized by lymphoid cells with open chromatin, diffuse infiltration into the surrounding mucosa, or mixtures with eosinophils and neutrophils) are frequently associated with acute rejection. The significance of lymphocytic cryptitis (increased numbers of lymphocytes in the crypt epithelium) is unclear. Although cryptitis is present in some cases of acute rejection, it is also observed in biopsy tissues without ACR (such as those exhibiting nonspecific enteritis, viral infections, or PTLD). Statistical analyses in this study failed to demonstrate a correlation between lymphocytic cryptitis and the diagnosis of acute rejection. Acute cryptitis (increased numbers of neutrophils in the crypt epithelium) is usually associated with various causes of acute enteritis and is not a diagnostic criterion for acute rejection.

TRANSPLANTATION

Adequate tissue sampling is necessary for accurate histologic diagnosis. Because the distribution of acute rejection may be patchy, multiple biopsies (usually three to five) are often required. Biopsies from either the ileum or the jejunum are sufficient for histologic evaluation in most cases, although sampling from both the ileum and the jejunum may be required in some cases with ambiguous diagnoses. The tissue obtained should be fixed in 10% neutral buffered formalin for at least 1 hr before processing, and multiple sections (usually 10–15) should be examined for each biopsy.

Differentiation between indeterminate and mild ACR is important for treatment planning. In our center, most of the histologically diagnosed mild acute rejection episodes were treated with increased immunosuppression (except when rejection occurred in association with opportunistic infections or PTLD), whereas treatment for indeterminate rejections was liberal, based on clinical assessments. A histologic distinction between these two categories can usually be made with this grading system. Among the listed criteria, the number of apoptotic bodies is most helpful (<6 apoptotic bodies per 10 crypts for indeterminate ACR versus >6 apoptotic bodies per 10 crypts for mild ACR), followed by perivenular infiltration (less common for indeterminate ACR and more common for mild ACR). We observed that mild acute rejection was associated with favorable clinical outcomes, which likely reflects successful immunosuppressive therapy. Indeterminate for acute rejection was also associated with favorable clinical outcomes, which likely reflects the minimal activity of acute rejection in this group and the use of immunosuppressive therapy for some of the patients.

Various pathologic conditions must be differentiated from acute rejection, the most common of which include nonspecific enteritis, CMV infection, EBV infection, and PTLD. Acute enteritis is often attributable to bacterial or viral infection and is characterized by neutrophil-rich infiltration in the lamina propria, with acute cryptitis but usually without significantly activated lymphocytes or increased apoptosis. CMV enteritis can sometimes be associated with increased inflammatory infiltration and increased apoptosis, and the diagnosis is made with the identification of characteristic nuclear and cytoplasmic viral inclusions, with confirmatory immunohistochemical staining. EBV infections and PTLD are often associated with significant mononuclear infiltration, and the diagnosis is made with the identification of atypical lymphoid cells on hematoxylin-eosin-stained sections, immunohistochemical staining for T and B cells, in situ hybridization for Epstein-Barr virus-encoded RNA, and clonality analysis. Ischemia-reperfusion injury is generally not a problem in the differential diagnosis, because it usually occurs immediately after reperfusion, with characteristic histologic features that resolve within 2 to 3 days in most cases. For patients with delayed recovery from severe ischemia-reperfusion injury, the diagnosis of early superimposed acute rejection can sometimes be difficult. Under such conditions, the presence of activated lymphocytes and eosinophils, ongoing crypt damage, and significant crypt apoptosis suggests acute rejection.

CONCLUSION

This study provides a reliable, predictive histopathologic schema for assessment of the severity of human small bowel acute rejection. The availability of this grading system should provide important guidance for effective immunosuppressive treatment of patients who undergo small bowel transplantation. It should also facilitate information exchange within and between transplantation centers.

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Banff Schema for Grading Liver Allograft Rejection: An International Consensus Document

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A panel of recognized experts in liver transplantation pathology, hepatology, and surgery was convened for the purpose of developing a consensus document for the grading of acute liver allograft rejection that is scientifically correct, simple, and reproducible and clinically useful. Over a period of 6 months pertinent issues were discussed via electronic communication media and a consensus conference was held in Banff, Canada in the summer of 1995. Based on previously published data and the combined experience of the group, the panel agreed on a common nomenclature and a set of histopathological criteria for the grading of acute liver allograft rejection, and a preferred method of reporting. Adoption of this internationally accepted, common grading system by scientific journals will minimize the problems associated with the use of multiple different local systems. Modifications of this working document to incorporate chronic rejection are expected in the future. (HEPATOLOGY 1997;25:658-663.)

The success of hepatic transplantation has resulted in its widespread use for treatment of many patients with endstage liver disease; it is currently offered by more than 100 centers worldwide. One-year survival rates range from 70% to 90%; and long-term survival of 50% to 60% of patients is not uncommon.[1] Therefore, an increasing number of physicians, including pathologists, many of whom have no specific training in transplantation biology, will become involved in the care of organ allograft recipients.

Despite the good short-term and acceptable long-term survival after hepatic transplantation, the morbidity associated with long-term immunosuppression is significant and rejection remains a persistent, but usually manageable, problem. Clinical research to improve patient survival and lessen morbidity is, therefore, inherent to the clinical practice of hepatic transplantation. Because patient follow-up and successful application of developments could be simplified by a common scale of recognizing, naming, and grading the severity of acute liver allograft rejection, members of an international consensus panel recently agreed upon a common nomenclature and set of definitions.[2] The group next agreed to create an internationally acceptable grading system, which has already been developed for kidney,[3] heart,[4] and lung.[5] At the Third Banff Conference on Allograft Pathology, a group of specialists in liver transplantation from North America, Europe, and Asia met for this purpose.

DEFINITION OF ACUTE REJECTION

In general, organ allograft rejection can be defined as, "an immunological reaction to the presence of a foreign tissue or organ, which has the potential to result in graft dysfunction and failure."[2] This report is specifically concerned with acute rejection, recently defined by the international consensus document on terminology for hepatic allograft rejection[2] as, "inflammation of the allograft, elicited by a genetic disparity between the donor and recipient, primarily affecting interlobular bile ducts and vascular endothelia, including portal veins and hepatic venules and occasionally the hepatic artery and its branches."[2] Early rejection, cellular rejection, nonductopenic rejection, rejection without duct loss, and reversible rejection are synonyms for acute rejection that appear in the literature, but their use is discouraged. The general clinical, laboratory, and histopathological abnormalities listed below were derived from the international consensus document.[2]

CLINICAL AND LABORATORY FINDINGS

Viewed from a biological perspective, any recipient's immune system will likely be perturbed after transplantation, resulting in immune activation.[2] However, viewed from a clinical perspective, because of baseline immunosuppressive therapy only some recipients manifest clinical symptoms of allograft recognition with, in the case of liver transplantation, liver biochemical abnormalities (most often), or frank hepatic dysfunction.[2] Therefore, it is important to distinguish between "biological" and "clinically relevant" rejection. The latter may require additional immunosuppressive treatment, although the distinction is not always achievable and treatment philosophies differ at various centers. This is particularly true for hepatic allografts, which are widely acknowledged to be unique. They are more resistant than others to humoral rejection, and are accepted without immunosuppressive therapy in some small and large experimental animal species. Of potential importance for human transplantation is the observation that in all animals in which a liver allograft is eventually accepted without drugs, the allograft undergoes a transient acute rejection crisis.[6-9] Thus, it should be understood that the histopathological diagnosis of acute rejection may not automatically signal that treatment is indicated, particularly if it is low grade. Adoption of a standardized histopathological grading system possibly could help determine if, and at what point, the histopathological severity of rejection can predict the need for, and success of antirejection
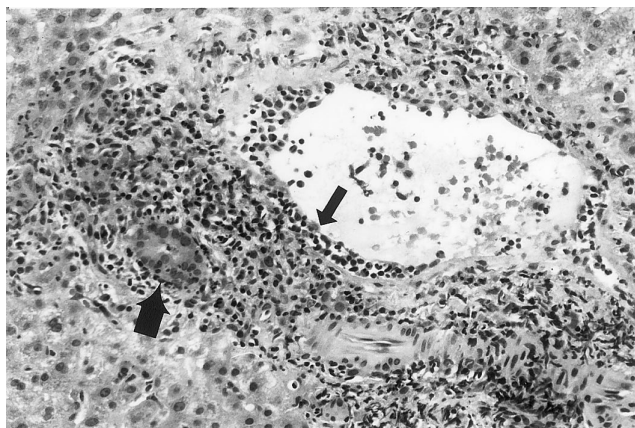
FIG. 1. Grading of acute rejection assumes the diagnosis has already been established: this portal tract shows all three of the typical histopathological features, two of which are required to make the diagnosis. There is: 1) a portal inflammatory infiltrate containing blastic lymphocytes and eosinophils; 2) subendothelial localization of the inflammatory cells in a portal vein branch (small arrow), and 3) inflammation and damage of small bile ducts (large arrow). If the subendothelial inflammation similar to this was present in most or all of the portal and/or hepatic venules, an RAI score of 2 for venous endothelial inflammation would be assigned.

therapy (see "Clinicopathological Correlation and Treatment of Acute Rejection").

When clinically apparent, acute rejection is usually first recognized between 5 and 30 days after transplantation. Earlier or later presentations can be seen in patients that receive less than therapeutic baseline immunosuppression. The clinical findings in early phases of mild acute rejection are often absent, although in late or severe cases, clinical findings include fever as well as swelling, cyanosis, and tenderness of the allograft. Bile often becomes pale in color and the flow is decreased. Occasionally, ascites develops because of liver swelling with increased intrahepatic pressure.[2]

Liver dysfunction, when present, usually manifests as concomitant nonselective elevations of the results of some or all of the standard liver injury tests, including total bilirubin, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, and alkaline phosphatase.[2] Leukocytosis and eosinophilia are also frequently present. Unfortunately, all clinical and laboratory findings lack sensitivity or specificity. The diagnosis is considered on clinical grounds and confirmed by examination of a core needle biopsy specimen. Some centers find that fine-needle aspirates of the allograft are useful adjunct.

HISTOPATHOLOGIC FINDINGS

Most investigators have observed similar histopathological findings associated with acute rejection.[10-22] Core needle biopsy specimens may show the following: 1) mixed but predominantly mononuclear portal inflammation, containing blastic (activated) lymphocytes, neutrophils, and frequently eosinophils; 2) bile duct inflammation/damage; and 3) subendothelial inflammation of portal veins or terminal hepatic venules.[2] At least two of these three features are required for a histopathological diagnosis of acute rejection (Fig. 1). Biochemical evidence of liver damage manifests as increased results of tests for liver injury, usually elevation of serum γ-glutamyl transpeptidase and alkaline phosphatase activities, are also frequently present. The diagnosis is strengthened if > 50% of the ducts are damaged or if unequivocal endotheliitis of portal vein branches or terminal hepatic venules can be identified. Occasional cases show mild mononuclear inflammation of the perivenular regions with only focal portal

tract changes. Additional findings such as ductopenia, spillover/piecemeal necrosis, eosinophilia, lobular inflammation, perivenular necrosis, arteritis, and inflammatory bridging, have been used in some systems for histopathological grading (see below).

Treatment of acute rejection with additional immunosuppression before a biopsy specimen is obtained may make the histopathological diagnosis more difficult, because of subsequent loss of the subendothelial infiltration of veins and of eosinophils, and a relative decrease in the number of mononuclear inflammatory cells.

GRADING OF ACUTE LIVER ALLOGRAFT REJECTION (CRITIQUE OF CURRENTLY POPULAR SYSTEMS)

The panel reviewed each system and agreed that the consensus scheme should fulfill the following criteria: scientific correctness, clinical relevance, simplicity, and reproducibility. They also recognized the need for flexibility and future modifications and therefore proposed a working formulation format for the current document.

The grading system used in Pittsburgh[23] is derived from those developed for kidney allograft.[24] It is based on the concept that serious injury from rejection is related to vascular compromise and ischemia, which can morphologically manifest as inflammatory or necrotizing arteritis and/or parenchymal necrosis and hemorrhage. The grading system developed in Minnesota by Snover et al.[19] is more specific to the liver and is based on a combination of an estimate of the severity of the inflammation and the presence and severity of damage or loss of key structures targeted for injury, such as the arterial vasculature or bile ducts. The above two systems have the advantage of simplicity[19, 23] and rely on pathophysiological concepts validated in renal transplantation. Prognostic significance has been shown at a single center.[19] Unfortunately, some of the features used in these schemes to define severe rejection are rarely found, poorly reproducible, or present so frequently in nonrejection complications that their usefulness in grading scheme is limited.[25] For example, while inflammatory or necrotizing arteritis[19, 23] represents a serious injury to the allograft, reproducibly identifying it in core needle biopsies is problematic.[25] In contrast, ballooning of perivenular hepatocytes[19] is frequently present in nonrejection graft syndromes and may not imply serious graft injury from an immunological insult. Bile duct loss, which has also been used to identify severe acute rejection more accurately reflects chronic rejection and possibly, a stage rather than a grade of rejection.

Kemnitz et al.[20,26] have devised a scheme similar to those mentioned above. However, increased emphasis is placed on precise numerical estimates of lobular injury, such as the percentage of necrosis, which may be difficult to reproduce and may not necessarily reflect rejection-related injury. Moreover, none of the systems was tested for reproducibility.

The European grading system for acute liver allograft rejection, developed by Hubscher and Dousset et al. at Birmingham[27-29], is based on a semiquantitative analysis of the diagnostic triad of Snover et al.[18] In this system, portal inflammation, bile duct damage, and venous endothelial inflammation are each graded semiquantitatively on a scale of 0 (absent) to 3 (severe). The individual scores are then added to produce an overall rejection score of 0 to 9, which is then converted to a rejection grade as follows: 0 to 2 = no rejection, 3 = borderline (consistent with), 4 to 5 = mild, 6 to 7 = moderate, and 8 to 9 = severe acute rejection. This system offers the attractive feature of quantifying the necro-inflammatory activity, as has recently become popular in the reporting and follow-up of patients with chronic hepatitis.[30-33] It also shows a good correlation between histological severity and clinical biochemical signs of graft dysfunction.[29] However, no obvious prognostic value has been shown.

The Royal Free Hospital, London (RFH) grading system[34] consists of a semiquantitative assessment of the diagnostic features of rejection, defined as immunosuppression responsive inflammation of rejection type, and identified by discriminant analysis. Mixed portal inflammation, eosinophils, endotheliitis, and bile duct damage were found to be independent, statistically significant contributors to the histological diagnosis of acute rejection. Each of the features are scored on a scale of 0 to 3, as in the European grading system, and a total score is derived by adding the individual scores together. Apart from the inclusion of eosinophils, which are of known diagnostic[35,36] and pathophysiological significance[37,38] as a separate variable in the RFH scheme, it is virtually identical to the European grading system. Like the European system, the RFH system offers a quantitative scale for the rejection-related activity, and is reproducible at the home institution.[34] However, neither the European system, nor the RFH system has been shown to have prognostic significance and the numerical cutoff points corresponding to the different degrees of rejection (and consequent therapeutic thresholds) need to be validated. In addition, there are no studies of inter-institutional scoring reproducibility.

The recently published scheme by the National Institute of Diabetes and Digestive Diseases and Kidney Diseases[39] had the advantages of being reproducible with prognostic significance documented at several centers. Unfortunately, the imprecise language used to explain the cutoffs for moderate and severe rejection makes the system difficult to follow, even for those experienced in the field.

INTERNATIONAL GRADING SYSTEM FOR ACUTE LIVER ALLOGRAFT REJECTION (RECOMMENDATIONS OF THE PANEL)

Grading of Rejection. The grading of rejection, as with hepatitis,[33] is a measure of the severity of the necro-inflammatory process. In addition, because rejection is more vasculocentric and vasculodestructive than hepatitis, some estimate of vascular or ischemic damage is needed to assess the full extent of the insult. This can be accomplished either by a global assessment of the biopsy using a "gestalt"[25] approach, or semiquantitatively with the assignment of numerical scores to different histopathological parameters. No data support one approach over the other, and in practice the two methods yield similar results (see below). Moreover, the semiquantitative approach could complement the global assessment by offering a greater degree of precision, by forcing the pathologist to critically evaluate important histopathological features. Conversely, the global approach can temper the semiquantitative analysis in cases with active inflammation and high scores, in which there is little architectural damage.

The panel agreed that existing grading systems for acute liver allograft rejection are conceptually similar, and that like chronic hepatitis, frequent monitoring and reporting of disease activity is an important function of biopsy analysis.[30-33] Therefore, in coming to a consensus, the panel drew upon the strengths, hopefully avoided the pitfalls, and corrected the weaknesses of the currently available grading systems. Portal inflammation, bile duct damage, subendothelial inflammation of portal veins, and terminal hepatic venules, strictly defined inflammatory or necrotizing arteritis and eosinophils (in the proper context) are features that the panel members regard as diagnostic of acute rejection. Portal inflammation, bile duct damage, strictly defined arteritis, and possibly confluent perivenular necrosis associated with perivenular inflammation are features that may also have prognostic significance, based on previous publication,[19,39] or personal experience. However, arteritis, as well as other findings such as bile duct loss, interstitial hemorrhage, and perivenular necrosis without inflammation are not included in the scheme, because they are poorly reproducible findings, considered to be part of chronic rejection, or also encountered frequently in nonrejection-related complications, respectively. If strictly defined arteritis can be shown to be a reproducible observation and present in more than a rare case, the current system can be modified to include it.

Being aware of the need for acceptability and thus simplicity, the panel agreed on a verbal grading of acute rejection based on the overall appearance of the biopsy according to the criteria listed in Table 1 (Fig. 2). It should be re-emphasized however, that any grading of acute rejection already presupposes that the diagnosis has been established. For example, use of the "indeterminate" category of acute rejection should be restricted to cases that have minor degrees of cellular infiltration that could possibly represent low grade or early acute rejection, but fail to meet the minimal diagnostic criteria. "Indeterminate" should not be used for cases in which one is unsure whether the inflammation is related to some other condition, such as chronic hepatitis C (see Complicating Conditions). After the global assessment, three specific features, portal inflammation, bile duct inflammation/damage, and venular inflammation, can be more critically evaluated and semiquantitatively scored on a 0 to 3 (mild, moderate, and severe) scale, according to the criteria listed in Table 2. The three are then added together to arrive at a final Rejection Activity Index (RAI) (Table 2), similar to the scoring developed for chronic hepatitis.[30-33] Modifications of the above system [19, 22, 29, 39] were made to arrive at a consensus scheme, so that features given the highest scores on the semi-quantitative analysis were the same as those shown to be of prognostic significance using the overall approach.

Potential problems using this method however, include: 1) the global assessment of rejection may under or overestimate the severity based on a semi-quantitative analysis and 2) the greater degree of "precision" achieved semiquantitatively may occur at the expense of reproducibility. We think that these pitfalls are unlikely to occur because both processes measure the same parameters or endpoints. Moreover, evaluation of a series of 50 posttransplantation liver allograft biopsy specimens using both methods by one of us (AJD) showed no significant differences between the systems. The reproducibility of the semiquantitative analysis will be the subject of future study by this group. The RAI, like other semiquantitative assessments of necro-inflammatory activity, is particularly attractive when evaluating new drugs or other treatment protocols and for comparison with previous biopsy specimens. Thus, it will be most valuable at academic centers involved with new developments in the field. Although strongly recommended for routine patient care, it is not required for day-to-day use if the pathologist chooses otherwise.

TABLE 1. Grading of Acute Liver Allograft Rejection

Global Assessment*	Criteria
Indeterminate	Portal inflammatory infiltrate that fails to meet the criteria for the diagnosis of acute rejection (see text)
Mild	Rejection infiltrate in a minority of the triads, that is generally mild, and confined within the portal spaces
Moderate	Rejection infiltrate, expanding most or all of the triads
Severe	As above for moderate, with spillover into periportal areas and moderate to severe perivenular inflammation that extends into the hepatic parenchyma and is associated with perivenular hepatocyte necrosis

NOTE. Global assessment of rejection grade mode on a review of the biopsy and after the diagnosis of rejection has been established.

* Verbal description of mild, moderate, or severe acute rejection could also be labeled as Grade I, II, and III, respectively.

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FIG. 2.   (A) Low power photomicrograph of a failed liver allograft with severe acute rejection. Note the prominent portal tract (PT) and central vein (CV) inflammation, associated with confluent perivenular necrosis, which is shown at a higher magnification in (B). These findings would elicit a diagnosis of severe acute rejection. (C) In the same liver allograft, the bile duct inflammation and damage was widespread, and there was focal lumenal disruption, eliciting an RAI score of 3 for bile duct damage (Table 2). Both the portal and venous endothelial inflammation were also scored as severe, or "3," resulting in a total RAI score of 9/9. (D) Sections from the hilum of this failed allograft also revealed clear cut necrotizing arteritis (arrow), which is rarely detected with certainty in needle biopsies.

Staging of Rejection. Staging of a biological phenomenon is performed in an attempt to codify a process that is largely unidirectional and evolves in a predictable pattern over a relevant period of time. Acute liver allograft rejection is, for the most part, widely considered to be a completely reversible phenomenon. In the uncommon event of allograft failure from acute rejection, the evolution is relatively rapid. Therefore, acute rejection is not readily amenable to staging. Chronic rejection on the other hand, usually evolves more slowly and is often, but not always,[40,41] unidirectional or irreversible. At this time it is not clear whether acute and chronic rejection represent the ends of a spectrum of alloreactivity, or if they are completely different biological processes. Considerable data suggest the former, because both processes appear to be triggered by alloreactivity, and persistent or severe acute rejection can result in allograft failure from chronic rejection.

Clinicopathological Correlation and Treatment of Acute Rejection. As alluded to in the introductory sections, the histopathological diagnosis of acute rejection does not necessarily imply that the rejection is clinically significant or requires treatment with increased immunosuppression. In fact, Schlitt et al.[42] have shown that up to 40% of patients in whom a biopsy shows acute rejection, according to the criteria of Snover et al.[18,19] did not have clinically apparent graft malfunction or significant elevations of results of liver injury tests, and did not require additional immunosuppressive therapy. Similar conclusions were also reached in a study

from Birmingham, in which 70% of histologically mild rejection episodes received no additional immunosuppression, without any adverse outcome.[28,29] A survey of the panel members showed no clear-cut consensus on the therapeutic approach to mild acute rejection (RAI $\leq$ 4) as defined in this report. In contrast, most centers report that patients with histopathological moderate or severe rejection (RAI $\geq$ 6) experience significant elevations of liver injury tests and the vast majority probably should, and usually are treated with additional immunosuppression. At present, no therapeutic recommendations can be inferred from the mild acute rejection grade, although some centers have exercised the option of routinely obtaining a follow-up biopsy after 1 to 2 weeks.

Complicating Conditions. Liver allografts are frequently affected by more than one condition. In the first few weeks after transplantation, preservation-related changes and mechanical problems with the vascular and/or biliary tree are the conditions that most commonly co-exist with acute rejection. Separation of the necro-inflammatory and ischemic damage of rejection from the same type of nonrejection insults is at times problematic, but achievable for the most part. For example, perivenular necrosis can occur in both preservation injury and severe rejection. However, the concomitant presence of mononuclear perivenular inflammation, portal changes of rejection, and absence of perivenular necrosis in a prior biopsy, are features that help to distinguish between the two. In contrast, more than several months after

TABLE 2. Rejection Activity Index

Category	Criteria	Score
Portal Inflammation	Mostly lymphocytic inflammation involving, but not noticeably expanding, a minority of the triads	1
	Expansion of most or all of the triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils and eosinophils	2
	Marked expansion of most or all of the triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the periportal parenchyma	3
Bile Duct Inflammation Damage	A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear:cytoplasmic ratio of the epithelial cells	1
	Most or all of the ducts infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity and cytoplasmic vacuolization of the epithelium	2
	As above for 2, with most or all of the ducts showing degenerative changes or focal lumenal disruption	3
Venous Endothelial Inflammation	Subendothelial lymphocytic infiltration involving some, but not a majority of the portal and/or hepatic venules	1
	Subendothelial infiltration involving most or all of the portal and/or hepatic venules	2
	As above for 2, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis	3

NOTE. Total Score = Sum of Components. Criteria that can be used to score liver allograft biopsies with acute rejection, as defined by the World Gastroenterology Consensus Document.

transplantation, chronic viral hepatitis and recurrence of autoimmune chronic inflammatory disorders pose considerable difficulties in differential diagnosis and with grading or scoring of rejection related activity.

The problem of differentiating duct damage associated with complicating conditions such as viral hepatitis C from that seen in acute rejection can be minimized by applying strict diagnostic criteria: damage of more than an occasional bile duct, the presence of unequivocal endotheliitis, and absence of significant lobular disarray and necro-inflammatory activity favor a diagnosis of acute rejection. However, problematic cases will still be encountered, and implicit in any grading scheme for acute rejection (including this one), is the notion that grading can be reliably applied to biopsies only when rejection is thought to be the sole or predominant cause of graft damage. Therefore, in cases where other causes of cellular infiltration are suspected, neither the overall grade nor the scores can be reliably applied. In such cases, it is left to the judgment of the pathologist whether apportioning the necro-inflammatory activity to rejection or other concurrent conditions is appropriate.

CONCLUSIONS AND RECOMMENDATION

Although the adequacy of any particular biopsy is ultimately left to the judgment of the pathologist, the panel recommends that at least two hematoxylin and eosin stained sections from at least two different levels, of a core needle biopsy containing at least five triads be examined. The ade-

quacy of the biopsy in the absence of any diagnostic findings when fewer than five portal tracts are identified, is again left to the pathologist's judgment.

The following format for the grading and reporting of acute liver allograft rejection is recommended, although all of this information is not needed in every case. The type of specimen and time after transplantation, if available, should be listed first. This is followed by the histopathological diagnosis(es). Although not necessary, some pathologists may prefer to list first the diagnosis perceived to be of greatest significance, followed by the second most important, and so forth. However, a comment on the presence or absence of acute rejection should be given for every biopsy, either in the diagnosis or comment section. This is followed by reporting of an RAI. The presence of chronic injury, such as bile duct loss or obliterative arteriopathy should also be listed. Lastly, a comparison with the most recent previous biopsy should be made if the pathologist feels that such a comparison is warranted. The following are several examples:

1. Liver allograft, needle biopsy (7 days posttransplantation)
   (a) Moderate preservation injury
   (b) No evidence of rejection (RAI = 0)
   (c) No previous biopsy for comparison

2. Liver allograft, needle biopsy (10 days posttransplantation)
   (a) Acute rejection, moderately active (RAI = 7)
   (b) Significantly worse than previous biopsy (S95-999 of 02/06/95{RAI = 2})

3. Liver allograft, needle biopsy (10 weeks posttransplantation)
   (a) Acute hepatitis, viral type C
   (b) No rejection (RAI = 0)

4. Liver allograft, needle biopsy (18 months posttransplantation)
   (a) Chronic hepatitis, viral type B, moderately active (HAI = 14)
   (b) Acute rejection, mildly active (RAI = 4)
   (c) Duct loss in 5/9 portal triads, suggestive of chronic rejection

We believe that this system will be easy to use and useful for physicians caring for allograft recipients. There already are data available to suggest that it will be both reproducible and have prognostic significance,[39] yet flexible enough to incorporate future development like the inclusion of chronic rejection or staging of rejection. We urge scientific journals to adopt this reporting system, classification, and grading of liver allograft rejection, to overcome the obstacles presented by the multiple schemes that currently exist and facilitate comparisons among different centers.

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A0248

ORIGINAL ARTICLES

CLINICAL HEART TRANSPLANTATION

Functional and Morphological Findings in Heart Transplant Recipients with a Normal Coronary Angiogram: An Analysis by Dobutamine Stress Echocardiography, Intracoronary Doppler and Intravascular Ultrasound

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Background: Coronary angiography is still the routine screening method for cardiac allograft vasculopathy in most transplant centers. This study was designed to analyze functional and morphologic changes in heart transplant recipients with normal angiographic findings.

Methods: Dobutamine stress echocardiography and intracoronary ultrasound were obtained in 56 patients with a normal coronary angiogram 41 ± 31 months after heart transplantation. Intracoronary Doppler flow velocity measurements before and after intracoronary adenosine administration were performed in 34 of 56 patients. Any regional wall motion abnormalities detected by stress echocardiography were regarded as abnormal. By quantitative intracoronary ultrasound analysis using a 6-grade scale, a mean grade of all coronary segments >3.0 was defined as significant intimal hyperplasia.

Results: Only 17 patients (30%) showed both a normal dobutamine stress echocardiogram and absence of significant intimal hyperplasia by intravascular

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ultrasound. Abnormal findings were observed in 39 patients (70%): both by dobutamine stress echocardiography and intravascular ultrasound in 22 patients, by intravascular ultrasound alone in 11 patients, and by dobutamine stress echocardiography alone in 6 patients. Coronary flow velocity reserve did not discriminate between patients with normal or abnormal intravascular ultrasound or dobutamine stress echocardiographic findings.

Conclusions: Only a minority of heart transplant patients with a normal coronary angiogram is free of pathological changes, when assessed by intravascular ultrasound and dobutamine stress echocardiography. Coronary flow velocity reserve does not seem useful to further characterize these patients. J Heart Lung Transplant 1999; 18:391–398.

Cardiac allograft vasculopathy (CAV) is one of the most common causes of death in the long-term course after heart transplantation.[1–3] The diagnosis of CAV is still mainly based on invasive methods, most commonly coronary angiography.[2] Coronary angiography, however, may not detect early changes, in particular when diffuse concentric luminal narrowing is present.[1–5] Intravascular ultrasound (IVUS) allows for direct investigation of vessel wall morphology and has emerged as the most sensitive invasive tool for identification of early stages of CAV in vivo.[2,3,6–9] In addition, several studies employed intracoronary Doppler flow analysis to assess the functional significance of CAV.[10–12] Among noninvasive tests, dobutamine stress echocardiography has been reported as the most promising method for the detection of CAV,[13–15] but is not yet part of the standard screening for CAV at many institutions. The aim of this study was to investigate the diagnostic significance of a normal coronary angiogram after heart transplantation in comparison with functional and morphological analysis by dobutamine stress echocardiography, intracoronary Doppler and intravascular ultrasound.

METHODS
Patient Population

From 100 consecutive orthotopic heart transplant recipients undergoing scheduled routine coronary angiography in the chronic postoperative phase ($\geq$10 months after transplantation), 56 patients were selected fulfilling the criteria of A) a normal coronary angiogram by visual analysis and B) an adequate echocardiographic image quality. The mean age was 49 ± 11 years, the postoperative interval was 41 ± 31 (10–144) months. Immunosuppressive therapy consisted of cyclosporine ($n = 46$), tacrolimus ($n = 10$), prednisone ($n = 46$) and azathioprine ($n = 30$). Antihypertensive treatment was received by 51 patients (angiotensin converting enzyme inhibitors, $n = 37$; diuretics, $n = 33$; calcium channel blockers, $n = 28$). All patients had given informed consent to the study protocol, that was approved by the University of Munich Ethics Committee.

Coronary Angiography

Cardiac catheterization and right ventricular endomyocardial biopsy were performed via the femoral approach within 24 hours of stress echocardiography. Coronary angiograms were obtained in standard orthogonal views after nitroglycerin administration using 7F or 8F catheters and a nonionic contrast agent. A visual analysis was performed by two investigators familiar with the specific problems of CAV (Klauss and Mudra). Only patients with a normal coronary angiogram were included in this study; any angiographic changes suggesting CAV as minimal luminal contour irregularities, slow flow of the contrast agent or narrowing of small vessels were classified as abnormal. Discrepancies between observers led to exclusion of the respective patient.

Intravascular Ultrasound

The details of IVUS image acquisition and analysis at our institution have been described previously.[8,16] IVUS images were obtained from several coronary artery segments using a mechanic 30 MHZ system (CVIS Inc, Sunnyvale, California) and a motorized pull-back device of the imaging probe. Representative images from each segment were digitized. In frames chosen for analysis, the transducer was located centrally in the vessel and was positioned coaxial to the artery to obtain undistorted cross sectional images. The site with most severe disease within each segment was evaluated quantitatively (Rieber and Klauss) according to the degree and circumferential extent of intimal hyperplasia: Grade 1, no intimal hyperplasia; grade 2, intimal thickness <0.3 mm, circumferential extent <180°; grade 3,

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<0.3 mm, $\geq180°$; grade 4, 0.3–0.5 mm, $<180°$; grade 5, 0.3–0.5 mm, $\geq180°$; and grade 6, >0.5 mm, 0–360°.[8,15] From all segments analyzed in each individual patient a mean grade was calculated. Based on previous observations,[15] a mean IVUS grade >3.0 was regarded as marker of relevant transplant vasculopathy. Intimal hyperplasia of this extent has been shown to be clearly abnormal.[17] An index of intimal area (intimal index) was calculated as (intimal area/vessel cross sectional area) $\times 100$.

Intracoronary Doppler

Coronary blood flow velocity measurements were performed using a 0.014-inch Doppler guide wire (FloWire^{TM}, Cardiometrics Inc, Mountain View, California). The Doppler guide wire was placed in a stable position within the proximal or mid segment of the left anterior descending artery or the left circumflex artery, depending on ease of access, and the Doppler signal was optimized. Average peak flow velocity was measured at baseline and after an intracoronary bolus injection of 16 μg adenosine, as previously described.[16] Coronary flow velocity reserve was calculated as ratio of hyperemic to baseline average peak flow velocity.

Dobutamine Stress Echocardiography

Vasoactive medication was withheld on the day of the stress test. After baseline evaluation, dobutamine infusion was started at 5μg/kg body weight/minute and increased stepwise by 5 μg to a maximum of 40 μg/kg/minute at 5 minute intervals.[15] Two-dimensional image sequences were obtained of the parasternal long and short axis and apical two and four chamber views. Regional wall motion was graded by experienced observers (Spes, Schnaack, Tammen) as normal or hyperkinetic (score 1), hypokinetic (score 2), akinetic (score 3), or dyskinetic (score 4) using a 16-segment-model. A normal response to dobutamine stress was defined as hyperkinesis and increased systolic wall thickening.[15] A wall motion score index was calculated as the sum of scores for each segment divided by the number of analyzed segments.

  M-mode echocardiography was used to quantify systolic thickening of the interventricular septum and the left ventricular posterior wall. Systolic wall thickening, calculated as end-systolic minus end-diastolic divided by end-diastolic wall thickness, has been shown to be decreased—in particular during stress—in patients with allograft vasculopathy.[15,18] In a previous study from our laboratory[18] in a cohort of heart transplant recipients without CAV by an-

giography and IVUS and without other cardiac complications, normal values for M-mode echocardiographic parameters in heart transplant recipients have been defined. According to these results, systolic wall thickening was regarded abnormal, when the values were below the following thresholds: systolic thickening of septum at rest $<17.2\%$, posterior wall at rest $<41.6\%$, septum at maximum stress $<45.9\%$, posterior wall at maximum stress $<67.6\%$.[18] Left ventricular hypertrophy was defined as an end-diastolic wall thickness >12 mm of the septum and the left ventricular posterior wall, respectively.

STATISTICS

Data are given as mean $\pm$ standard deviation, unless stated otherwise. Unpaired Student′s t-test, Mann-Whitney U-test, or Fisher′s exact test were used, as appropriate, to assess differences between groups. Differences between stress stages were analyzed with a Wilcoxon rank sum test. A p value of <0.05 was considered significant.

RESULTS
Clinical Characteristics

Arterial hypertension requiring medical therapy was present in 51 of 56 patients (91%). The mean systolic / diastolic blood pressure at rest before stress echocardiography was $125 \pm 17/76 \pm 13$ mmHg. There were no differences in blood pressure in subgroups of patients with and without abnormal either echocardiographic or IVUS findings. All patients in this study were non-smokers. Fifteen patients (27%) had diabetes mellitus after transplantation; the prevalence of diabetes was not different in patients with or without functional or morphological abnormalities. Data on the lipid profile at the time of the study were available in 46 patients. Mean serum cholesterol was 230 ± 39 mg/dl, low-density lipoprotein cholesterol 138 ± 35 mg/dl, high-density lipoprotein cholesterol 62 ± 32 mg/dl, and serum triglycerides 173 ± 108 mg/dl. Again, no differences between subgroups were observed.

Coronary Angiography, Endomyocardial Biopsy

All 56 coronary angiograms were without evidence of CAV, as predefined by the study inclusion criteria. Endomyocardial biopsy was performed in 46 occasions and analyzed according to the ISHT classification;[19] 37 biopsies were free of rejection (grade 0), 9 showed evidence of mild acute rejection (grade 1A, $n = 5$, grade 1B, $n = 4$).

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DSE normal, mean IVUS grade ≤ 3.0

DSE normal, mean IVUS grade > 3.0

DSE abnormal, mean IVUS grade ≤ 3.0

DSE abnormal, mean IVUS grade > 3.0
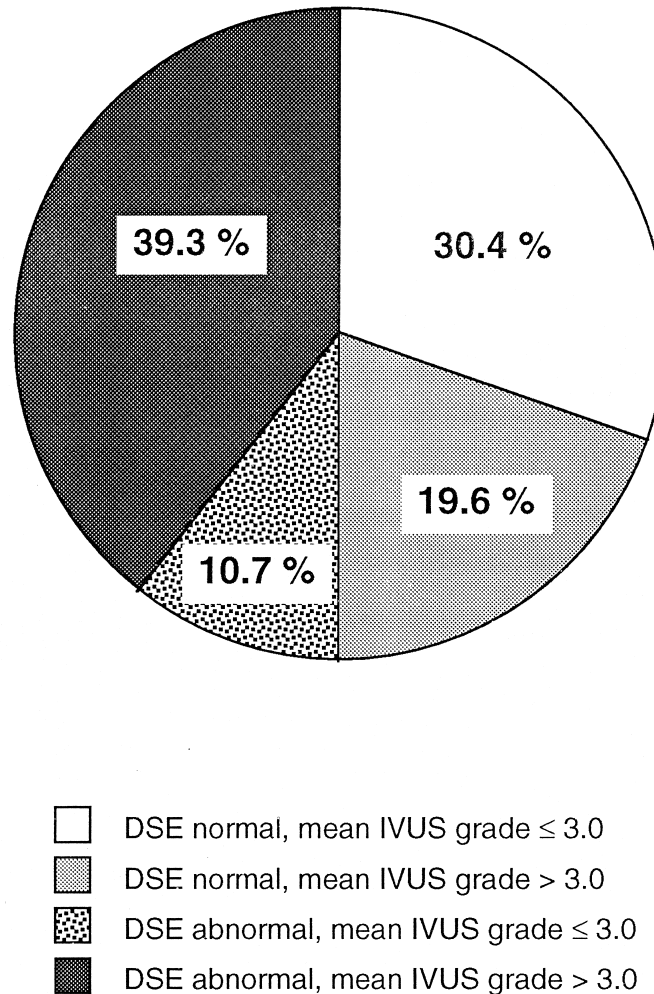
FIGURE   Frequency distribution of normal and abnormal findings by dobutamine stress echocardiography (DSE) and intravascular ultrasound. DSE abnormal denotes regional wall motion abnormalities by 2D-analysis or reduction of systolic wall thickening by M-mode echocardiography under the lower limit of normal.[18] Further explanation: see text.

Intravascular Ultrasound

In each patient, 1–3 (mean 1.5 ± 0.5) major epicardial arteries were studied by IVUS (in total, 82 epicardial vessels; left anterior descending artery, $n = 45$; left circumflex artery, $n = 31$; right coronary artery, $n = 6$). A total of 267 segments (mean 4.8 ± 1.4 segments/patient, range 3–8) were evaluated by IVUS. The mean IVUS grade of all coronary segments in each patient was 3.35 ± 1.62 (range 1.0–6.0), the mean intimal index 15.1 ± 11.5% (range 0–42.1%). The mean IVUS grade was >3.0 in 33 of 56 patients with normal angiograms (59%) (Figure).

Dobutamine Stress Echocardiography

No patient had angina during stress testing. The heart rate increased from 89 ± 14 beats/min at rest to 143 ± 14 beats/min ($p < 0.0001$) at maximum dobutamine infusion; the mean peak dobutamine dosage was 18 ± 5 µg/kg/minute (range 10–35). The rate-pressure product rose from 11174 ± 2344 to 19678 ± 4342 mmHg/minute ($p < 0.0001$).Technically adequate M-mode echocardiographic readings could be obtained in 53 of 56 patients. Systolic wall thickening was normal in 31 of 53 patients (58%). In 10 cases, normal thickening at rest decreased below normal at maximum dobutamine stress; 9 cases

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TABLE I Intracoronary Doppler measurements (mean $\pm$ standard error) in relation to dobutamine stress echocardiography (DSE) and intravascular ultrasound (IVUS)

DSE (all areas)/ IVUS (all segments)	DSE normal and IVUS grade ≤3.0	DSE abnormal or IVUS grade >3.0	DSE abnormal and IVUS grade >3.0
Number of patients studied			
LAD (n)	6	12	11
CX (n)	5	8	8
Average peak velocity (cm/s)			
LAD, rest	24 ± 3	17 ± 2	17 ± 2
LAD, adenosine	66 ± 5	57 ± 5	59 ± 6
CX, rest	19 ± 2	16 ± 1	17 ± 3
CX, adenosine	54 ± 6	53 ± 3	53 ± 7
Coronary flow velocity reserve			
LAD	2.82 ± 0.25	3.34 ± 0.21	3.63 ± 0.21
CX	2.86 ± 0.25	3.31 ± 0.19	3.13 ± 0.23

DSE abnormal denotes regional wall motion abnormalities by 2D-analysis or reduction of systolic wall thickening by M-mode echocardiography under the lower limit of normal.[18] IVUS ≤/>3.0 denotes the mean grading of all coronary segments analyzed within a patient. LAD/CX denote left anterior descending/left circumflex artery. None of the parameters differed between groups with a $p < 0.05$.

showed reduced thickening at rest and during stress; and 4 patients had impaired resting contractility, that normalized during dobutamine stress.

2D-Echocardiography was normal in 34 patients (61%). In 22 patients, regional wall motion abnormalities were observed: 5 patients with normal findings at rest had stress induced wall motion abnormalities in 2.8 ± 2.0 segments ($p < 0.04$ vs rest), the wall motion score index in these patients at maximum stress was 1.18 ± 0.16. In 9 patients, preexisting wall motion abnormalities at rest increased at dobutamine stress (5.4 ± 3.0 vs 6.3 ± 2.7 segments/patient, $p < 0.02$ vs rest; score index 1.26 ± 0.17 vs 1.48 ± 0.26, $p < 0.002$). In 4 patients, wall motion abnormalities at rest remained unchanged (2.0 ± 1.4 segments, score index 1.08 ± 0.07), and 4 patients with resting wall motion abnormalities (mean IVUS grade $\phi \leq 3.0$, $n = 2$; IVUS grade > 3.0, $n = 2$) showed an improvement during dobutamine infusion (4.2 ± 1.5 vs 3.5 ± 2.9 affected segments, NS; score index 1.27 ± 0.09 vs 1.15 ± 0.14, $p < 0.05$). Combined 2D- and M-mode echocardiographic analysis detected abnormalities in 28 patients (50%); 18 of these patients were abnormal by both echocardiographic methods, 4 cases by 2D-analysis only, and 6 patients by M-mode analysis of wall thickening only.

Intracoronary Doppler

Coronary flow velocity was studied in 34 patients. The left anterior descending artery was analyzed in 13 cases, the left circumflex artery in 5 cases and both arteries in 16 cases. When flow data were compared to functional data obtained from all ventricular segments and the mean IVUS grade of all coronary segments analyzed, average peak velocity at rest tended to be higher in patients without abnormal stress echocardiographic or IVUS findings (Table I). Due to a similar increase in all groups, the adenosine-stimulated flow velocity reserve was not different in patients with wall motion abnormalities or moderate-to-severe intimal hyperplasia, compared to patients without these changes (Table I). In addition, no differences in flow parameters were observed, when flow measurements were compared to mean the IVUS grade of the study vessel only and regional wall motion/wall thickening only in the left ventricular area related to the study vessel (Table II).The presence of mild acute rejection did not influence the flow velocity reserve. The flow velocity reserve of the left anterior descending artery and the left circumflex artery was not related to hypertrophy of the septum or the left ventricular posterior wall, respectively, in the small group of patients studied.

DISCUSSION

Coronary angiography has remained the most commonly used screening method for CAV.[2,3,5] Several reports exist, however, on rapid progression from a normal angiogram to severe vasculopathy, that in some cases of sudden death after heart transplantation was diagnosed only at necropsy.[4,20] IVUS has been demonstrated to be the most sensitive invasive tool for diagnosis of CAV in vivo.[6–10] IVUS, however, does not allow to investigate the complete coronary artery system as it is restricted to major

TABLE II   Intracoronary Doppler measurements (mean $\pm$ standard error) in relation to regional findings in the study vessel by intravascular ultrasound (IVUS) and in its perfusion area by dobutamine stress echocardiography (DSE)

	DSE normal and IVUS grade $\leq$3.0	DSE abnormal or IVUS grade >3.0	DSE abnormal and IVUS grade >3.0
Study vessel LAD: DSE (area supplied by LAD) IVUS - analysis LAD			
Number of patients	4	16	8
APV, LAD, rest (cm/s)	26 $\pm$ 4	17 $\pm$ 1	19 $\pm$ 3
APV, LAD, adenosine (cm/s)	65 $\pm$ 8	57 $\pm$ 4	62 $\pm$ 7
CFVR, LAD	2.71 $\pm$ 0.38	3.38 $\pm$ 0.17	3.52 $\pm$ 0.27
Study vessel CX: DSE (area supplied by CX) IVUS - analysis CX			
Number of patients	5	5	3
APV, CX, rest (cm/s)	20 $\pm$ 4	18 $\pm$ 1	17 $\pm$ 1
APV, CX, adenosine (cm/s)	65 $\pm$ 10	51 $\pm$ 5	48 $\pm$ 0
CFVR, CX	3.36 $\pm$ 0.27	2.79 $\pm$ 0.27	2.85 $\pm$ 0.10

Study vessel LAD: DSE: 2D - analysis of the left ventricular area usually supplied by the LAD and M-mode analysis of systolic thickening of the septum. IVUS $\leq$/>3.0 denotes the mean grading of all LAD segments analyzed. Study vessel CX: DSE: 2D - analysis of the left ventricular area usually supplied by the CX and M-mode analysis of systolic thickening of the left ventricular posterior wall. IVUS $\leq$/>3.0 denotes the mean grading of all CX segments analyzed. None of the parameters differed between groups with a $p < 0.05$.
LAD: left anterior descending artery; CX: left circumflex artery; APV: average peak velocity; CFVR: coronary flow velocity reserve.

epicardial vessels. Isolated analysis of IVUS may, therefore, not always detect CAV evident by angiographic analysis. The present study is the first to compare dobutamine stress echocardiography with IVUS and intracoronary Doppler in long-term heart transplant recipients with a normal coronary angiogram. The IVUS findings in this study confirm previous reports, that angiography is an insensitive method to detect early changes of CAV.[1–10] A normal coronary angiogram after heart transplantation, therefore, does not allow to exclude morphologic evidence of CAV. Dobutamine stress echocardiography, a useful noninvasive technique to detect angiographic 50–70% diameter stenosis of major epicardial vessels in coronary artery disease,[21] is gaining increasing interest in the follow-up of heart transplant patients.[13–15,18] In the present study, functional abnormalities were demonstrated by echocardiography in a high percentage of patients even with a normal angiogram. In addition to the most commonly used regional wall motion analysis by 2D-echocardiography, an impairment of systolic wall thickening could be detected by quantitative analysis of M-mode recordings. Wall motion abnormalities were observed in some patients without moderate to severe intimal hyperplasia in the vessels visualized by IVUS. Our data are in accordance with previous studies, that also observed wall motion abnormalities during dobutamine stress or exercise echocardiography in the absence of morphologic abnormalities in the allograft.[13–15,22] Wall motion abnormalities in the absence of lesions in the epicardial arteries may be a caused by endothelial dysfunction[10–12, 23–26] and small vessel disease,[27] that may be detected neither by angiography nor by IVUS. Absent or poor collateral vessels[5] in the setting of CAV may also cause functional abnormalities. This would imply that dobutamine stress echocardiography, by imaging the complete left ventricle, may be very useful to assess the functional reserve of the myocardium of the allografts. The method, however, does not allow to discriminate if impairment of myocardial function is due to abnormal function of the myocardium itself, interstitial changes (eg fibrosis) or due to alterated myocardial perfusion either in the epicardial arteries or in the microcirculation. Intracoronary Doppler flow measurements have been proposed to assess the significance of coronary artery stenoses and the integrity of the microcirculation. This technique has also been used in heart transplant patients. No correlation, however, of morphologic intimal changes of epicardial allograft arteries and coronary flow velocity reserve could be demonstrated in previous studies.[10–12] These data were confirmed in the present analysis. In this study, in addition, coronary flow velocity reserve after administration of the endothelium-independent vasodilator adenosine was compared with myocardial pump function, as assessed by dobutamine echocardiography. No relation, however, between wall motion abnormalities and flow velocity reserve could be found. Preliminary data by

other investigators have shown similar results.[28] Simultaneous analysis of flow velocity reserve and epicardial endothelial responses,[29] however, might provide further information on the relation between morphologic abnormalities, myocardial functional reserve and epicardial and endothelial function. Clinical characteristics were not different in subgroups of patients with or without abnormal functional or morphologic findings in the present study; this is possibly explained by the small sample size of the respective subgroups. Both IVUS[30] and dobutamine stress echocardiography[31–33] may provide prognostic information in heart transplant recipients. Coronary flow velocity reserve, however, did not identify patients with abnormal findings by IVUS or dobutamine stress echocardiography in transplant recipients with a normal coronary angiogram. The value of Doppler coronary flow velocity measurements to predict the clinical outcome in these patients has to be determined in further investigations.

CONCLUSION

A normal angiogram alone does not exclude relevant CAV in heart transplant recipients. Despite of a normal coronary angiogram, the majority of patients in this study had functional alterations as regional wall motion abnormalities and impaired systolic wall thickening by dobutamine stress echocardiography and morphologic evidence of CAV in the epicardial arteries by IVUS. Measurement of coronary flow velocity reserve, however, does not seem useful to identify patients with abnormal findings by IVUS or dobutamine echocardiography and can, therefore, not substitute these methods.

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Assessment of cardiac allograft vasculopathy late after heart transplantation: when is coronary angiography necessary?

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ALLOGRAFT VASCULOPATHY

Assessment of Cardiac Allograft Vasculopathy Late After Heart Transplantation: When Is Coronary Angiography Necessary?

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Background: Cardiac allograft vasculopathy (CAV) represents a major prognostic factor in long-term survivors of heart transplantation (HTx). Reliable diagnosis of CAV late after HTx is important but remains the domain of invasive techniques such as coronary angiography.

Methods: To test alternative approaches, 54 consecutive HTx recipients (mean time since HTx: 52 months) were studied with intravascular ultrasound (IVUS), angiography, dobutamine stress echocardiography and immunofluorescence staining against anti-thrombin III (AT-III) in endomyocardial biopsies. Univariate and multivariate predictors as well as receiver-operating-characteristic (ROC) curves of different sets of predictors were calculated.

Results: Using IVUS as reference standard, CAV was present in 80% of subjects. Coronary angiography identified CAV correctly in only 44% of cases. If AT-III staining alone was used as a diagnostic criterion, CAV was correctly identified in 77% of subjects. In a multivariate analysis, only AT-III, donor age and echocardiography at rest emerged as independent predictors of CAV ($p < 0.05$ for all), yielding an excellent discriminative power.

Conclusions: With almost equal reliability when compared with IVUS, CAV can be identified using information on donor age, wall motion score at rest and AT-III staining late after HTx. Coronary angiography may be limited to patients with a high probability score and should not be used routinely for surveillance of CAV. J Heart Lung Transplant 2006;25:1103–8. Copyright © 2006 by the International Society for Heart and Lung Transplantation.

Cardiac allograft vasculopathy (CAV) is a major prognostic factor determining morbidity and mortality among the growing number of long-term survivors of heart transplantation (HTx).[1,2] Five years post-transplant, angiographically detectable CAV is prevalent in 42% of survivors.[3] Management of CAV consists of symptomatic treatment for left ventricular dysfunction and re-vascularization procedures. In more advanced disease, re-transplantation remains the only curative

approach.[4] Early detection of CAV facilitates risk stratification and early initiation of treatment thus potentially enhancing graft survival and patient outcome.[2]

Histologically, CAV is characterized by diffuse concentric intimal hyperplasia affecting extramural coronary arteries but also arterioles, venules and capillaries.[5] The donor endothelium, the border between the immune systems of donor and recipient, is thought to play a pivotal pathophysiologic role in the development and clinical course of CAV.[5] Markers of endothelial activation and function, such as anti-thrombin III (AT-III), assessed early after transplantation by immunohistochemistry, have been reported to correlate significantly with the development and progression of CAV as well as graft failure due to CAV.[5–7] Histologic CAV testing in heart transplant recipients can be performed without additional patient discomfort because right ventricular endomyocardial biopsies are routinely obtained at regular time intervals in many transplant centers even late post-operatively.

Intravascular ultrasound (IVUS) has provided important insights into the in vivo morphology of CAV and is the accepted reference standard to visualize and diagnose CAV.[8,9] In contrast, coronary angiography is only moderately sensitive although reasonably specific for CAV diagnosis[10]; however, it imposes a small risk and some discomfort to the patient, may cause kidney

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damage after contrast agent application, and consumes resources. Nevertheless, due to its lower costs and a definite, albeit small additional risk of IVUS,[11] coronary angiography is more widely used for CAV screening and follow-up during routine transplant surveillance.[12]

More recently, non-invasive echocardiographic imaging in conjunction with dobutamine stress echocardiography (DSE) has been shown to detect functionally relevant CAV with a sensitivity and specificity similar or even superior to coronary angiography.[13] However, due to imaging problems this method may not be reliably applied in all patients. Because coronary angiography underestimates the prevalence of CAV and the limited availability and applicability of IVUS,[10,13] additional diagnostic certainty is needed.

The present study aimed to assess whether pathologic AT-III immunostaining within the cardiac microvasculature is suited to predict the presence of CAV. We further estimated the incremental value of pathologic AT-III immunostaining in comparison or in addition to non-invasive DSE, coronary angiography and clinical variables.

METHODS

Fifty-four consecutive heart transplant recipients (22% female; age, 49.2 ± 11.5 years; time since transplantation, 52 ± 37 [median, 36; range, 11 to 75] months) were investigated upon routine post-transplant monitoring. DSE was done on the day of admission, and coronary angiography, IVUS and right ventricular endomyocardial biopsy were performed in one session on the next day. The local university ethics review board reviewed the study and all patients gave informed consent to participate.

Coronary angiography data were analyzed by two blinded expert observers using a qualitative grading system: Grade I, normal angiogram; Grade II, luminal irregularities or diameter reduction <30%; Grade III, diameter reduction between 30% and 50%; and Grade IV, diameter reduction ≥50% and/or diffuse narrowing of small vessels. CAV was considered present if angiographic changes of Grade II or greater were seen.[13]

IVUS images were obtained using a standardized motorized pullback system (2.9F, 30 MHz, CVIS, Inc., Sunnyvale, CA). The site with the most severe disease in each segment was graded according to the degree and circumferential extent of intimal hyperplasia on a 6-grade scale. A mean IVUS grade was calculated from all segments analyzed in each patient as described elsewhere.[13] The diagnosis of CAV was made if the mean IVUS grade was >3.0.[13]

Echocardiography was performed at rest and under maximal dobutamine-induced stress and analyzed for identification of functionally relevant presence of CAV as described elsewhere.[13] In brief, standard M-mode

parameters were measured to assess left ventricular dimensions and function. From B-mode images a wall motion score was calculated using a 16-segment model. Wall motion in each segment was graded as: normal/hyperkinetic; hypokinetic; akinetic; or dyskinetic (score 1 to 4). A wall motion score of 16.0 at rest and stress was considered normal. Results from 2-dimensional (2D) echocardiography were classified as abnormal if any wall motion abnormalities were detected.[13]

Immunohistochemical studies were performed on right ventricular endomyocardial biopsy samples obtained during cardiac catheterization for rejection surveillance, as described elsewhere.[14] In brief, biopsy samples were blocked in OCT compound (Tissue-Tek, Miles Scientific, Naperville, IL) and snap frozen in liquid nitrogen. Cryostat sections (6 μm) were cut in a cryotome (SLEE, Mainz, Germany) at −28°C and allowed to air-dry overnight. The primary antibody directed against AT-III (Dako Diagnostika, Hamburg, Germany) was identical to the antibody employed by Labarerre et al,[15] diluted with 10% fetal calf serum (FCS) in phosphate-buffered saline (0.05 mol/liter, pH 7.4). A fluorescein-isothyocyanate–conjugated rabbit–anti-mouse secondary antibody (Dako Diagnostika), diluted 1:50 in 10% FCS, was used.

Negative control experiments were performed by omitting the primary antibody. Further, a single specimen exhibiting normal AT-III immunostaining was used with each staining procedure as positive control. The presence of immunohistochemical reactivity to the antigen was evaluated from coded photomicrographs projected in a darkened room by two independent investigators blinded to the origins of the biopsy specimens, as previously reported.[14] Presence of AT-III in arterial and arteriolar smooth muscle cells, arterial intima and venous endothelium was considered a normal pattern of vascular AT-III. Absence of vascular AT-III was considered an abnormal pattern,[16] whereas presence of capillary AT-III was disregarded because the role of AT-III in capillaries remains unclear. For statistical analysis, fluorescence intensity was Grade 0 (undetectable fluorescence, i.e., pathologic AT-III staining) or Grade 1 (detectable fluorescence, i.e., normal AT-III staining). In the case of disagreement between observers (photomicrographs of 3 patients), grading was achieved by consensus. Endomyocardial biospies with histologically proven rejection were excluded from analysis.

Data Analysis

Data are presented as mean ± standard deviation (SD), unless stated otherwise. The Mann-Whitney U-test and chi-square test were used to test differences between sub-groups with and without CAV, as appropriate. Logistic regression was used to estimate the univariate

association between baseline characteristics and presence of CAV, with a liberal alpha ($p < 0.15$). Univariate predictors were tested in a multivariate model using the change-in-estimate criterion in a backward selection procedure,[17] and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. To assess the incremental contribution of the multivariate predictors, the areas under the receiver-operating-characteristic (ROC) curve of different sets of predictors (models) were compared.[18] Differences in the discriminative value between models were estimated by differences in ROC area, taking into account the correlation between models, as they were based on the same cases.[19] Statistical analysis was performed using SPSS, version 11.5.1.

RESULTS

Presence of CAV was diagnosed in 43 of 54 (80%) subjects using the IVUS criterion of Grade >3.0 (mean). The study characteristics are given in Table 1 for all patients and for sub-groups with and without CAV. Patient characteristics, including the immunosuppressive regimen and the concomitant medication, were comparable between these sub-groups except for donor age, which was higher in the CAV patients (Table 1). Subsequent steps in the diagnostic work-up for CAV are shown in Table 2. Systolic blood pressure and the rate–pressure product at rest were significantly higher in the CAV group. Both echocardiography at rest and at maximal dobutamine stress showed a significantly worse wall motion score in subjects with CAV. Coronary angiography

correctly identified CAV in only 44% (19 of 43) of cases, whereas 81% of CAV-negative patients were correctly classified. AT-III staining of Grade 0 was observed in 36 of 54 (67%) subjects. Thus, CAV was diagnosed correctly in 77% of the patients in whom IVUS identified CAV (33 of 43). In 3 CAV-negative subjects, pathologic AT-III staining was observed (Table 2); however, the mean IVUS grade in these false-positive subjects was quite high, specifically 3.0, 2.8 and 2.5.

Univariate predictors of CAV according to IVUS criteria were AT-III, wall motion score, rate–pressure product at rest, wall motion score at maximal stress, donor age, systolic blood pressure at rest, angiographically defined lumen abnormality, and fractional shortening at maximal stress (listed by decreasing strength of association ($p < 0.15$ for all). In the multivariate model, only AT-III (OR: 8.80, 95% CI: 1.96 to 19.59), donor age per 10 years (OR: 2.60, 95% CI: 1.10 to 6.16) and wall motion score at rest (OR: 3.89, 95% CI: 1.63 to 18.41) emerged as independent predictors of CAV ($p < 0.05$ for all).

Donor age was a powerful estimator of CAV diagnosis that, in itself, yielded an area under the ROC curve of 0.76 (Table 3). Adding the result of wall motion analysis at rest significantly increased the area under the ROC curve to 0.86. Further addition of information on stress testing or the angiographic result did not improve the area. By contrast, adding information on AT-III immunohistochemistry increased the area further, yielding an excellent discriminative power of 0.92 (95% CI: 0.85 to 0.99; Table 3).

Table 1. Patient Characteristics in the Whole Cohort and in Sub-groups Without and With Cardiac Allograft Vasculopathy

	All patients ($n = 54$)	IVUS ≤3 ($n = 11$)	IVUS >3 ($n = 43$)	p^{a}
Age of host, years	49 (11)	50 (11)	49 (11)	NS
Female gender of host, n (%)	12 (22)	2 (18)	10 (23)	NS
Age of donor, years	31 (12)	23 (9)	33 (12)	0.011
Diabetes, n (%)	12 (22.2)	3 (27)	9 (21)	NS
Pre-transplant cardiac diagnosis				
Dilated cardiomyopathy, n (%)	32 (59)	7 (64)	25 (58)	NS
Ischemic cardiomyopathy, n (%)	17 (32)	4 (36)	13 (30)	NS
Others, n (%)	5 (9)	0 (0)	5 (12)	NS
Immunosuppressive treatment				
Cyclosporine, n (%)	43 (80)	8 (73)	35 (81)	NS
Tacrolimus, n (%)	10 (19)	3 (27)	7 (16)	NS
Azathioprine, n (%)	30 (56)	5 (46)	25 (58)	NS
Prednisolone, n (%)	47 (87)	8 (73)	39 (91)	NS
Concomitant medication				
ACE inhibitor, n (%)	37 (69)	8 (73)	29 (67)	NS
Calcium-channel blocker	31 (57)	6 (55)	25 (58)	NS
Lipid-lowering drug, n (%)	17 (32)	1 (9)	16 (37)	0.071
Acetylsalicylic acid, n (%)	10 (19)	2 (18)	8 (19)	NS

Data are mean (SD), unless indicated otherwise. IVUS, intravascular coronary ultrasound; ACE, angiotensin-converting enzyme.
   [a]Chi-square test or Mann–Whitney U-test used for comparisons between sub-groups with and without cardiac graft vasculopathy, as appropriate.

Table 2. Diagnostic Procedures Performed to Confirm or Exclude Cardiac Allograft Vasculopathy

	All patients ($n = 54$)	IVUS ≤ 3 ($n = 11$)	IVUS >3 ($n = 43$)	p^a
Echocardiography at rest				
Heart rate, beats per minute (bpm)	92.0 (13.0)	89.0 (13.7)	92.8 (12.9)	NS
Systolic blood pressure, mm Hg	124.8 (18.1)	114.1 (17.4)	127.6 (17.4)	0.011
Diastolic blood pressure, mm Hg	78.3 (12.0)	73.6 (12.9)	79.5 (11.7)	NS
Rate–pressure product, mm Hg/min · 1,000	11.5 (2.4)	10.0 (1.4)	11.9 (2.5)	0.012
Fractional shortening, %	34.8 (6.3)	35.5 (5.9)	34.6 (6.5)	NS
Wall motion score	18.0 (3.5)	16.1 (0.3)	18.5 (3.8)	0.004
Echocardiography indicative of CAV, n (%)	26 (48)	1 (9.1)	25 (58.1)	0.004
Echocardiography at maximal dobutamine dose				
Heart rate, bpm	142.3 (13.6)	143.1 (16.5)	142.1 (12.9)	NS
Systolic blood pressure, mm Hg	136.8 (26.8)	124.1 (28.6)	140.1 (25.6)	0.078
Diastolic blood pressure, mm Hg	59.5 (14.0)	55.5 (14.0)	60.6 (14.0)	NS
Rate–pressure product, mm Hg/min · 1,000	19.4 (4.2)	17.6 (3.5)	19.9 (4.2)	0.108
Fractional shortening, %	47.8 (7.8)	51.8 (3.6)	47.0 (8.1)	0.078
Wall motion score	18.5 (3.9)	16.4 (0.9)	19.0 (4.1)	0.019
Dobutamine stress test indicative of CAV, n (%)	26 (48)	2 (18.2)	25 (58.1)	0.039
Summary information from echocardiography at rest and maximal stress indicative of CAV, n (%)	29 (53.7)	2 (18.2)	27 (62.8)	0.015
Coronary angiography				
Coronary angiogram indicative of CAV, n (%)	20 (37)	1 (9.1)	19 (44.2)	0.039
Intracoronary ultrasound (IVUS)				
Mean IVUS grade	4.2 (1.5)	2.0 (0.8)	4.8 (1.0)	<0.00001
Presence of CAV, n (%)	43 (79.6)	0 (0.0)	43 (100.0)	—
Immunohistochemistry in endomyocardial biopsy				
Pathologic AT-III immunostaining (i.e., Grade 0), n (%)	36 (67)	3 (27)	33 (77)	0.004

Data are mean (SD), unless indicated otherwise. IVUS, intravascular coronary ultrasound; CAV, cardiac allograft vasculopathy; AT-III, anti-thrombin III.
[a] Chi-square test or Mann–Whitney U-test used for comparisons between sub-groups with and without cardiac graft vasculopathy, as appropriate.

DISCUSSION

The main finding of this study is that, after a mean of 4 years post-HTx, CAV is present in 80% of cases according to IVUS criteria, and that CAV as detected by IVUS can be reliably identified using information on donor age, wall motion score at rest and AT-III staining. Compared with wall motion score at stress and qualitative coronary angiography, only AT-III staining significantly increased the diagnostic accuracy. Our data underscore the potential of immunohistochemical AT-III staining as a diagnostic tool for routine surveillance of CAV in heart transplant recipients.

Although the pathophysiologic mechanisms leading to CAV are not thoroughly understood, it is known from serially obtained EMB early after heart transplantation that endothelial injury (e.g., reperfusion damage) may enhance the development and progression of CAV.[6,20] AT-III is a key component of the heparin sulfate proteoglycan–anti-thrombin natural anti-coagulant pathway as it accelerates thrombin neutralization in endothelial and smooth muscle cells. Endothelial injury promotes loss of thrombo-resistant properties as indicated by AT-III depletion in arterial and arteriolar smooth muscle cells, arterial intima and venous endothelium. AT-III depletion within the cardiac microvasculature has been associated with early development of CAV and subsequent allograft failure.[7,21] This study was performed to

Table 3. Incremental Diagnostic Value of Different Sets of Predictors of Cardiac Allograft Vasculopathy

Model	(Set of) predictor(s)	Area under the ROC curve (95% CI)	p
1	Donor age	0.76 (0.61–0.92)	<0.001[a]
2	Donor age + wall motion score at rest	0.86 (0.74–0.96)	<0.05[b]
3	Donor age + wall motion score at rest + angiography	0.86 (0.77–0.95)	NS[c]
4	Donor age + wall motion score at rest + wall motion score at stress	0.85 (0.74–0.96)	NS[c]
5	Donor age + wall motion score at rest + AT-III	0.92 (0.85–0.99)	<0.05[c]

Receiver operating characteristic (ROC): a value of 0.5 for the area under the ROC curve indicates diagnostic indecision, similar to flipping a coin; a value of 1.0 indicates perfect discrimination.
[a] p vs chance value of 0.5.
[b] p vs Model 1 (NS, not statistically significant).
[c] p vs Model 2.

test the diagnostic potential of immunohistochemical AT-III, angiography, clinical variables and non-invasive functional imaging late after HTx. To the best of our knowledge, this is the first report on the independent and incremental value of AT-III staining to identify CAV in long-term heart transplant recipients, when combined with information on donor age and echocardiography at rest.

Treatment of CAV represents a major problem, although some progress has been made.[22,23] Interestingly, anti-thrombotic treatment with heparin has proven beneficial in experimental models. Data obtained in a rat heterotopic heart model suggests that treatment with cyclosporine and low-molecular-weight heparin reduces frequency and severity of CAV and graft rejection, although the influence of this therapy on AT-III levels was not investigated.[24] Respective evidence in clinical heart transplantation is lacking.

IVUS is the accepted reference method for early detection and monitoring of CAV. However, IVUS is an invasive procedure that is performed during cardiac catheterization, and it is costly, carries a small risk, and is not readily available at all transplant centers.[11,12] Most transplant centers traditionally perform periodic coronary angiography to screen for the presence of CAV. However, in the present study, only 44% of patients with IVUS-confirmed CAV were correctly identified by coronary angiography alone. This is in line with previous studies indicating systematic underestimation of the prevalence of CAV by coronary angiography.[9,10] Therefore, it appears desirable to achieve reliable CAV monitoring using a set of parameters consisting of clinical characteristics, non-invasive functional testing, and an immunohistochemical analysis of a biopsy specimen.

In the present study, donor age was a powerful determinant of CAV. Because others have also reported this strong association,[3] it appears reasonable to always use the information on donor age in models with CAV diagnosis.[13] Combining information on donor age and echocardiography expectedly increased the diagnostic accuracy (area under the ROC curve = 0.86), because a pathologic wall motion score at rest indicates significant coronary artery disease. Remarkably, most of the patients with IVUS-proven CAV exhibited abnormal wall motion already at rest; thus, the additional diagnostic yield of stress echo was insignificant in this cohort late after transplantation. Addition of angiography did not further improve the predictive value of the model. In contrast, adding AT-III staining to donor age and wall motion score at rest significantly increased the area under the ROC curve, yielding an excellent discriminative power. Thus, AT-III staining emerged as a clinically relevant and useful marker in a population at high risk for CAV late after HTx.
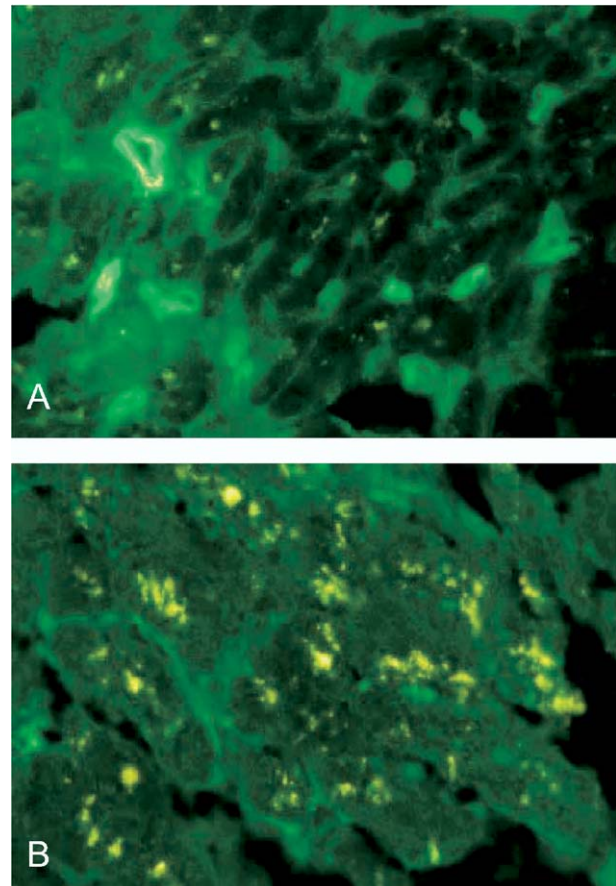
Figure 1. Representative AT-III staining in right ventricular endomyocardial biopsy specimens of two different patients (original magnification ×400). (A) AT-III Grade 1 staining. No evidence of CAV. The image shows bright yellow staining in arterioles and venules; the faint capillary AT-III immunostaining was disregarded (see text). (B) AT-III Grade 0 staining. AT-III depletion in arterioles and venules: presence of CAV as confirmed by IVUS. Bright yellow staining represents lipofuscin background staining. Staining in blood vessels identical to negative control experiments.

Limitations

AT-III staining was measured only once late after transplantation. Thus, we were unable to demonstrate a cause-and-effect relationship between early loss of AT-III and subsequent development of CAV in our study population. Further, the prognostic implications of these findings are not known, and a long-term prospective follow-up of a larger cohort of patients is required for absolute risk prediction. Still, a high-risk score as an indicator of developing CAV should increase efforts for vigorous control of traditional risk factors.

In conclusion, the combination of echocardiography at rest, donor age, and loss of AT-III staining in right ventricular endomyocardial biopsies in long-term survivors after heart transplantation was found to be a strong predictor for the presence of CAV as defined by IVUS criteria. AT-III staining yielded significant incremental

information complementary to that obtained by established and widely employed diagnostic tools such as coronary angiography and echocardiography. Coronary angiography appeared insensitive for diagnosis of CAV in this cohort of late post-operative patients and may therefore be limited to patients with a high risk as assessed by donor age, echocardiography and AT-III staining, instead of being used routinely for long-term CAV surveillance. (Figure 1).

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REVIEW

Evaluation of coronary allograft vasculopathy using multi-detector row computed tomography: a systematic review

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Summary

Coronary allograft vasculopathy (CAV) is a significant cause of morbidity and mortality after cardiac transplantation and requires frequent surveillance with catheter-based coronary angiography (CCA). Multi-detector row computed tomography (MDCT) has been shown to be effective in assessing atherosclerosis in native coronary arteries. This article systematically reviews the literature to determine the accuracy of MDCT in CAV assessment. An English-language literature search was performed using EMBASE, OVID, PubMed, and Cochrane Library databases. Studies that directly compared MDCT with CCA and/or IVUS for the detection of coronary artery stenosis or significant intimal thickening in cardiac transplant patients were analyzed. Data were pooled to obtain weighted sensitivities, specificities, and diagnostic accuracies. Negative and positive predictive values (NPV/PPV) were calculated. A total of seven studies with a sum of 272 patients were included in this review. There were three studies examining 16-slice MDCT and four studies looking at 64-slice MDCT in CAV. Using per-segment analysis, MDCT assessed between 91% and 96% of all coronary segments when evaluating for stenosis. Pooled estimates for sensitivity and specificity for MDCT ranged from 82% to 89% and 89% to 99%, respectively, while NPV was 99%. Per-patient analysis revealed a sensitivity of 87–100% and NPV of 96–100%. PPV was less than 50% for 64-slice MDCT in both per-segment and per-patient analysis. When compared with IVUS, MDCT had a sensitivity of 74–96% and specificity of 88–92% in assessment of intimal thickening. NPV and PPV were 80–81% and 84–98%, respectively. The high sensitivity and NPV of MDCT suggest that it may be a useful, noninvasive screening tool to rule out CAV.

Keywords: Coronary allograft vasculopathy • Multi-detector row computed tomography • Catheter-based coronary angiography • Intravascular ultrasound

INTRODUCTION

After the first year of transplantation, coronary allograft vasculopathy (CAV) is the leading cause of death among recipients of cardiac transplants [1]. At 5 years post-transplantation, CAV may be detected in up to 50% of patients [2]. CAV, resulting from endothelial damage due to immune and nonimmune factors, leads to luminal narrowing, myocardial ischemia, and eventually graft failure.

CAV is often clinically silent in the setting of denervation of 70–90% of cardiac allografts [3], making diagnosis difficult. Noninvasive tests are not sensitive or specific enough for assessment of CAV. Therefore, in most centers, catheter-based coronary angiography (CCA) is performed on an annual basis. In addition to being invasive, CCA also underestimates the presence of disease when compared with histopathologic analysis and intravascular ultrasound (IVUS) [4,5] due to the absence of focal lesions and positive remodeling associated with CAV [5,6]. IVUS is the gold standard for detection of CAV, but it is invasive, limited to large epicardial arteries, and costly, particularly if done on an annual basis.

Multi-detector row computed tomography (MDCT) may be a new, accurate noninvasive test for evaluating CAV. MDCT has

been demonstrated to be reliable for detection of stenosis in native coronary arteries, with particularly high sensitivities and negative predictive values (NPVs) [7]. Due to its ability to visualize luminal as well as mural abnormalities, MDCT may be able to detect CAV earlier than CCA much like IVUS. This systematic review attempts to determine the accuracy of MDCT in assessing CAV when compared with CCA and IVUS.

METHODS

A literature search was performed using the online databases MEDLINE, OVID, EMBASE, and Cochrane library with keywords describing MDCT assessment of CAV. Search terms were as follows: 'computed tomography' matched with 'coronary artery' and 'transplant' or 'allograft vasculopathy' or 'transplant vasculopathy'. We included studies that directly compared 16-slice or 64-slice MDCT (or dual-source computed tomography – DSCT) with CCA and/or IVUS for the detection of coronary artery stenosis or significant wall thickening in cardiac transplant patients. References from included studies were manually searched to supplement electronic searches (Fig. 1).
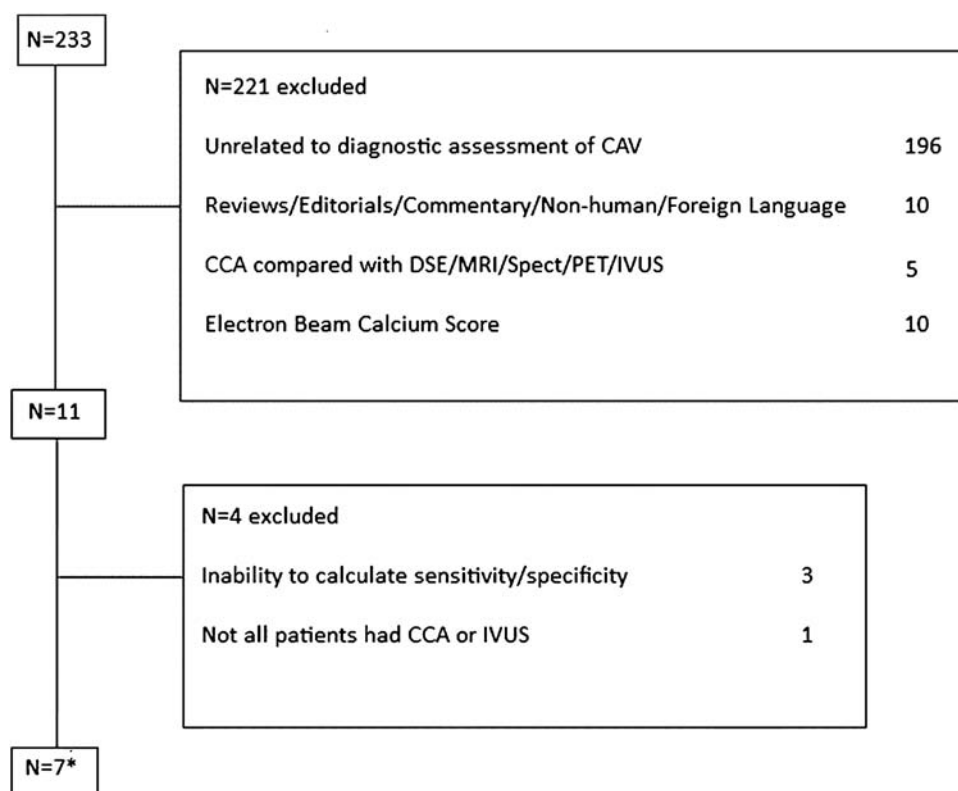
Figure 1: Flow diagram of literature search for MDCT assessment of CAV (performed on November 6, 2010). *All studies included for final pooled analysis compared 16-slice or 64-slice MDCT with a reference standard of CCA or IVUS. Sensitivity and specificity had to be noted or calculable from the data.

Original articles from the electronic database and manual search were reviewed, while all other forms of publication were excluded (e.g., case reports, letters, editorials, animal and *in vitro* studies, abstracts only, and case series with fewer than 10 patients). References from review articles were also examined to identify original research. In addition, all foreign-language articles were excluded. Articles which failed to provide head-to-head comparison between MDCT and the reference standard of CCA or IVUS were also not included.

Both per-segment and per-patient analysis for MDCT assessment of CAV was performed when possible. Coronary segments were described most often using the system proposed by the American Heart Association [8], or modified versions of this system. Significant stenosis was defined by the presence of a >50% or ≥50% obstructive lesion [9–14] in every study except one where >70% obstruction was used as a cutoff [15]. Early CAV, as visualized by IVUS, was defined as intimal thickening of >0.5 mm [12,14] or the presence of intimal thickening in the absence of obstructive coronary lesions (<50% stenosis) [13]. In studies included for analysis, the sensitivity and specificity of MDCT had to be noted or calculable from provided data. Data from all studies were pooled to obtain a weighted sensitivity, specificity, NPV, positive predictive value (PPV), and diagnostic accuracy for MDCT.

The quality of each study cited was reviewed using the quality information questionnaire from the University of Alberta Evidenced Based Working Group. This questionnaire has been used for selection of studies in previous MDCT-based guidelines [16]. Both authors reviewed each citation.

RESULTS

Study characteristics

There were seven studies included in the analysis of MDCT assessment of CAV [9–15]. In all investigations, the reason for patient assessment with the reference standard was annual surveillance of CAV. Exclusion criteria for most studies included renal dysfunction (creatinine > 106–212 µmmol l^{-1}) [10–15], atrial arrhythmias [10,13,14], and evidence of clinical instability [10–12,14]. β-Blockers were administered for heart rate reduction in only one 16-slice and two 64-slice studies [10–12] because transplant patients are often denervated and have decreased response to these medications. The efficacy of β-blockers was variable with two studies reporting reductions in heart rate by >10 beats min^{-1} with administration [11,12], while one study noted no significant change [10]. Only two investigations did not corroborate qualitative CCA assessment of coronary stenosis with quantitative coronary angiography (QCA) [9,11]. Observers were blinded to the results of the reference standard in each study analyzed.

Radiation doses were reported in only four of seven studies [11–14]. These doses ranged from 3 to 10 mSV [11,13] in 16-slice studies and 10 to 18 mSv [12,14] in 64-slice studies. Approximately 60–100 cm^3 of iodinated contrast was administered for both 16-slice and 64-slice MDCT [10–14]. The absence of contrast-induced nephropathy and contrast-associated allergic reaction was documented in five studies [10–14], but not reported in the remaining two studies [9,15].

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Table 1:   Baseline characteristics of cardiac transplant patients undergoing MDCT for assessment of CAV

	Authors	Patient numbers	Mean age (years)	Mean duration from transplantation (years)	Men (%)	BMI (kg m^{-2})	Mean HR
16-Slice MDCT	Romeo et al.	53	48	7.6	75	N/S	69.5 ± 11
	Sigurdsson et al.[a]	54	54	N/S	89	28.1	90 ± 12
	Pichler et al.[a]	66	58	8.5	91	26.8	87 ± 17
	Total	173					
64-Slice MDCT	Nunoda et al.[a,b]	10	35	N/S	77	N/S	N/S
	Gregory et al.	20	52	5.8	80	29.5	77 ± 7
	von Ziegler et al.	28	53	7.7	100	NS	86 ± 13
	Schepis et al.[a,c]	41	40	2.8	81	26	80 ± 15
	Total	99					

N/S: not specified.
[a] β-Blockers were not administered for heart rate reduction.
[b] Blinding was not specified.
[c] DSCT used for scanning.

Table 2:   Segment-based evaluation of 16-slice and 64-slice MDCT for the detection of CAV when compared with CCA

	Authors	Patient numbers	Per-segment prevalence of CAV (%)	Vessel diameter analyzed (mm)	Assessibility (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Diagnostic accuracy (%)
16-Slice MDCT	Romeo et al.[a]	53	2	>1.5	96	80	99	99	80	99
	Sigurdsson et al.	54	7	≥1.5	96	86	99	99	81	98
	Pichler et al.[b]	66	2	≥1.5	94	71	99	99	91	94
	Total	173	4		96	82	99	99	82	98
64-Slice MDCT	Nunoda et al.[a]	10	6	>1.5	100	40	100	97	100	97
	von Ziegler et al.	28	3	N/S	81	88	97	99	47	97
	Schepis et al.	41	16	≥1.5	96	93	80	98	48	83
	Total	79	10		91		89	89	99	49

N/S: not specified. Severe coronary artery stenosis was defined by >50% or ≥50% obstruction of lumen in the coronary artery tree of a patient after cardiac transplantation.
[a] Quantitative coronary angiography was not performed. Evaluation of coronary artery stenosis was made based on assessment with qualitative angiography.
[b] Severe coronary artery stenosis was defined by >70% obstruction of lumen in the coronary artery tree.

Baseline characteristics

A total of 272 cardiac transplant patients underwent MDCT for assessment of CAV (Table 1). Of these, 173 patients had 16-slice MDCT, while 99 underwent 64-slice MDCT [9–15]. Mean age for patients was between 35 and 58 years. Participants in these studies ranged from 77% to 100% male. MDCT scan was performed a mean of 2.8–8.5 years after transplantation [10–12,14,15]. Average heart rate for patients was between 69.5 and 90 BPM at the time of scanning [10–15]. Body mass index was 26–29.5 kg m^{-2}, but this was reported in only four of seven studies [12–15].

Assessment of significant CAV: comparing MDCT to CCA

There were three 16-slice and three 64-slice studies that directly compared MDCT with CCAin evaluating CAV (Table 2). When analysis was performed on a per-coronary segment basis, 16-slice and 64-slice MDCT were able to respectively assess 96% and 91% of all coronary segments visualized by CCA. In comparison with CCA, 64-slice MDCT had a sensitivity and specificity of 89%, similar to results obtained with 16-slice MDCT. 16-Slice and 64-slice MDCT had an NPV of 99%. The pooled PPV for 64-slice MDCT was poor at 49%, substantially lower than that observed with 16-slice MDCT.

Four studies provided sufficient data for per-patient assessment of MDCT efficacy in CAV (Table 3). Only one of these investigations used 64-slice MDCT. In this analysis, MDCT was able to assess between 84% and 93% of transplant patients for CAV. Sensitivity and specificity for MDCT ranged from 87% to 100% and 81% to 91%, respectively. NPV was notably very high at 96–100%. As with per-coronary segment analysis, PPV was markedly lower with 64-slice MDCT when compared with 16-slice MDCT.

Only three studies have examined the accuracy of MDCT in quantifying luminal stenosis. When compared with CCA as a reference standard, Pearson correlation coefficients ranged from 0.64 to 0.89, suggesting good correlation. When assessing the accuracy of measurements, the standard error of estimate in these studies was 14.4–15.0%.

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Table 3: Patient-based evaluation of 16-slice and 64-slice MDCT for the assessment of CAV when compared with CCA

	Authors	Patient numbers	Per-patient prevalence of CAV (%)	Vessel diameter analyzed (mm)	Assessibility (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Diagnostic accuracy (%)
16-Slice MDCT	Romeo et al.[a]	53	16	>1.5	83	71	97	93	83	92
	Sigurdson et al.	54	30	>1.5	98	94	79	97	65	83
	Pichler et al.[b]	66	18	≥1.5	73	88	97	97	88	95
	Total	173	18		84	87	91	96	73	90
64-Slice MDCT	von Ziegler et al.	28	19	N/S	93	100	81	100	56	85

N/S: not specified. Severe coronary artery stenosis was defined by >50% or >50% obstruction of lumen in the coronary artery tree of a patient after cardiac transplantation.
[a] Quantitative coronary angiography was not performed. Evaluation of coronary artery stenosis was made based on assessment with qualitative angiography.
[b] Severe coronary artery stenosis was defined by >70% obstruction of lumen in the coronary artery tree.

Table 4: Evaluation of 16-slice and 64-slice MDCT for the detection of early CAV when compared with IVUS

	Authors	Patient numbers	Prevalence of early CAV (%)	Assessibility (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Diagnostic accuracy (%)
16-Slice MDCT	Sigurdsson et al.[a]	54	32	99	96	88	80	98	91
64-Slice MDCT	Gregory et al.[b]	20	33	97	76	88	84	82	83
	Schepis et al.[b]	41	32	98	70	92	77	89	82
	Total	61	32	97	74	89	81	84	83

[a] Early CAV was defined as presence of intimal thickening in the absence of obstructive coronary disease (<50% luminal stenosis).
[b] Early CAV was defined by intimal thickening >0.5 mm.

Initial evaluation of early CAV: comparing MDCT to IVUS

Three studies compared the efficacy of MDCT with IVUS for the detection of intimal thickening due to CAV (Table 4). All studies used per-segment-based analysis and none provided sufficient data for per-patient analysis. Proximal coronary segments were predominantly analyzed. Only 19–36% of all potential segments visualized by CCA or MDCT were examined with IVUS. MDCT was able to assess between 97% and 99% of all segments analyzed by IVUS. 16-slice and 64-slice MDCT had a sensitivity of 74–96% and specificity of 88–89%. NPVs for MDCT were between 80 and 81%, while PPV ranged from 84% to 98%. The overall diagnostic accuracy for MDCT in assessing intimal thickening was 83–91%. There was no documented attempt to describe plaque characteristics, such as calcification, with MDCT or IVUS in any study.

DISCUSSION

Assessment of significant CAV: comparing MDCT to CCA

As cardiac denervation is frequent after transplantation, patients with CAV remain asymptomatic and commonly present with graft failure or malignant cardiac arrhythmias [17]. Therefore, CCA is performed annually as surveillance for CAV. However, the clinical utility of annual assessment has been questioned [18]. From a histopathologic viewpoint, CAV is associated with concentric intimal thickening, positive remodeling and distal disease, making diagnosis difficult on CCA [19]. CCA has been shown to have poor sensitivity for assessing CAV when compared with IVUS. [20] In addition, CCA carries a small (0.1%) but significant risk for stroke, myocardial infarction, or death. [21] These risks may increase in the setting of repeated testing as is the standard for transplant patients [18].

MDCT has recently been shown to accurately assess native CAD [7], suggesting that it may be a useful screening tool for detection of CAV. In addition to being noninvasive, MDCT may be able to evaluate both luminal and mural changes associated with CAV, unlike CCA. However, elevated heart rates seen in transplant patients lead to motion artifact and image degradation, potentially limiting the efficacy of MDCT in this setting. Patients with rapid heart rates were often excluded entirely from native CAD studies [7]. In a preliminary study involving pediatric cardiac transplant patient population, motion artifact prevented the analysis of 25% coronary segments with 4-slice MDCT [22]. Newer scanners have increased spatial and temporal resolution and may permit accurate detection of CAV despite increased heart rates.

A total of six studies compared the efficacy of 16-slice and 64-slice MDCT to CCA in assessing CAV. When analysis was performed on a per-coronary segment basis, both 16-slice and 64-slice MDCT were able to respectively assess 96% and 91% of all segments visualized by CCA. As in native CAD assessment,

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MDCT had good-to-excellent sensitivities, specificities, and NPVs when evaluating coronary disease in cardiac allografts compared to the reference standard of CCA. In particular, sensitivities of 82–89% and NPVs of 99% for MDCT on a per-coronary-segment-based analysis indicate that MDCT can reliably exclude CAV in transplant populations. This is emphasized further when looking at per-patient analysis of MDCT, which showed a sensitivity of 87–100% in detecting significant CAV, suggesting that between 8 and 9/10 patients may be able to avoid or delay initial screening CCA if MDCT was used as a gatekeeper to more invasive testing.

In comparison to MDCT assessment of native CAD, PPV was lower for 64-slice MDCT in the detection of CAV [7]. This was likely attributable to the low prevalence (18–19%) of CAV in this population. In the ACCURACY trial [23], MDCT had a similarly low PPV (51% with vessel-based analysis) in assessing significant native CAD where disease prevalence was also low (25%). The low PPV reinforces the limitations of MDCT as primarily a screening rather than confirmatory test for diagnosis of CAV. In addition, in the setting of low-disease prevalence commonly seen with referrals for MDCT, one or two false positives may substantially worsen PPV.

The reason for reduced assessment of coronary artery segments with 64-slice MDCT when compared with 16-slice MDCT remains unclear considering the improved spatial and temporal resolution associated with newer scanner technology. Although not obviously explained, this has been a finding described in previous meta-analysis examining the efficacy of both scanners [24]. No appreciable differences in heart rates, body mass index, or calcification were noted between the 16-slice and 64-slice study populations, although these indices were not reported in all studies (Table 1). The most plausible explanation for these results is better initial screening of patients who may be suitable for MDCT scanning in the 16-slice studies. In addition, one study looking at 64-slice MDCT did not report cutoff diameters for coronary segments assessed, and therefore may have attempted to examine vessels smaller than 1.5 mm which are still poorly visualized by current-generation scanners. The factors were amplified by the relatively small cumulative sample size of transplant patients undergoing MDCT, likely leading to unexpectedly better efficacy of 16-slice MDCT.

The accuracy of MDCT relies on image quality, which, in turn, is partially dependent on heart rate. Even with the improved temporal resolution of 64-slice scanners, heart rate has been shown to correlate inversely with image quality by causing motion artifact [25]. In the setting of considerably increased heart rates associated with cardiac transplant patients, MDCT was able to assess a high percentage of coronary segments and maintain good diagnostic accuracy for evaluation of CAV. However, in comparison to its assessment of native CAD, 64-slice MDCT had mildly reduced specificity and diagnostic accuracy when assessing CAV. Poor image quality of coronary segments was attributable to motion artifact in 21–61% of cases [12–14]; therefore, heart rate likely did have an impact on accuracy. To improve the efficacy of MDCT, newer technologies such as DSCT may allow for high-quality imaging, despite elevated heart rates. In the one study where DSCT was used for assessment of CAV, 96% of all coronary segments were deemed evaluable. No correlation was noted between DSCT image quality and heart rate [14]. In addition, the use of multisegment reconstruction algorithms has been proposed to reduce the effects of heart rate on MDCT efficacy [26].

In the transplant patient population, accurate quantification of luminal diameter is important to evaluate annual progression of CAV. In studies that compared the efficacy of MDCT in assessing the degree of luminal stenosis to QCA, Pearson correlation coefficients ranged from moderate to excellent ($r = 0.64$–0.89), suggesting good correlation between both modalities [10–12]. However, only three studies included quantification of luminal diameter assessments by MDCT. In addition, standard error of estimation in these studies was substantial (14.4–15.0%). Therefore, it remains unclear as to whether MDCT can monitor progression of CAV in patients with pre-existing disease.

Initial evaluation of early CAV: comparing MDCT to IVUS

IVUS is the preferred modality for assessing onset and progression of CAV [14]. It allows for evaluation of luminal diameter, as well as assessment of intimal and medial thickness unlike CCA. IVUS can detect CAV in greater than 50% of asymptomatic patients within 1-year posttransplantation, whereas CCA detects disease in only 10–20% of such patients [27]. Despite this, there are a number of limitations associated with IVUS, including an inability to evaluate the distal aspects of the coronary tree, as well as cost and risk of repeated procedures. The risk of serious complications associated with IVUS has been estimated at between 1% and 3% [28].

Like IVUS, MDCT allows for visualization of the coronary vessel lumen and wall. Due to its efficacy in delineating soft tissue accurately, MDCT can potentially assess both intimal thickening and plaque burden. In native CAD, pooled weighted analysis revealed MDCT had very good sensitivities of 87–92% and specificities of 81–86% at visualizing atherosclerotic plaques when compared with IVUS [29]. This suggests that MDCT may be an ideal noninvasive modality for assessment of early CAV (Fig. 2).

In the three studies comparing MDCT with IVUS for the detection of intimal thickening due to CAV, MDCT was able to visualize between 97% and 99% of all segments analyzed by IVUS. This is considerably higher than values obtained when MDCT was measured against CCA because only proximal coronary segments were assessed by IVUS. Compared to IVUS, both 16-slice and 64-slice MDCT had excellent sensitivity and specificity in evaluating intimal thickening in transplant patients. The NPVs for MDCT were between 77% and 84%, which further emphasized the utility of MDCT at ruling out early disease.

In addition, the PPV for MDCT in assessing CAV when compared with IVUS ranged from 82% to 98%, substantially higher than values obtained with CCA comparisons. This is again partially attributable to the evaluation of only large, proximal coronary vessels with IVUS. These results may also be due to the fact that there is comparatively little calcium deposition in CAV in contrast to native CAD [30]. In the assessment of native noncalcified plaques, MDCT has a tendency to underestimate plaque volume when compared to IVUS, resulting in fewer false positive tests [14]. Unfortunately, the prevalence of significant calcification in our pooled analysis could not be determined as it was not reported in any MDCT and IVUS CAV study.

Using IVUS as the reference standard, MDCT has been shown to have substantially better sensitivity (70% vs 11%) at detecting nonobstructive (<50% stenosis) CAV when compared with CCA
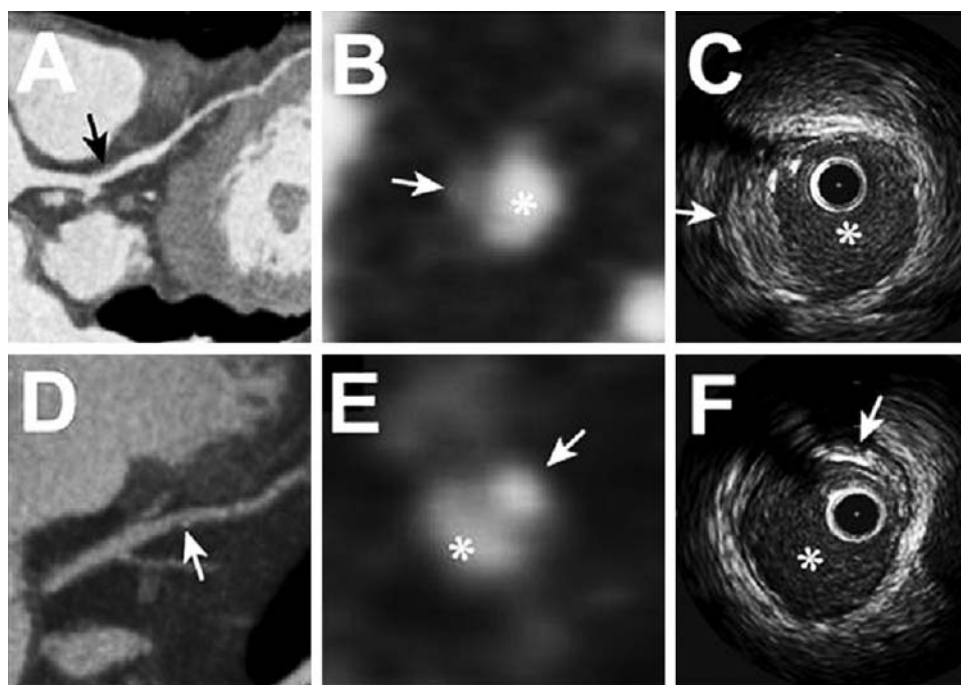
Figure 2: Correlation of CAV detected by MDCT and IVUS. (**A**) Curved multiplanar reformatted MDCT image and (**B**) multiplanar reformatted image of the vessel cross section demonstrates a noncalcified coronary plaque (*arrow*) and lumen (*asterisk*) of the proximal left anterior descending coronary artery. (**C**) IVUS image in the same location confirms presence of CAV (*arrow*). (**D**) Maximum intensity projection MDCT image and (**E**) multiplanar reformatted image of vessel cross section of the mid-left anterior descending coronary artery show a calcified nodule 'embedded' in the noncalcified plaque (*arrow*) and the lumen (*asterisk*). (**F**) An IVUS image in the same location confirms the presence of CAV with a calcified nodule (*arrow*). Reprinted with permission from Gregory et al. [12].

[12]. MDCT may be capable of detecting CAV-related wall thickening in up to 45% more coronary segments than CCA [11]. This again reinforces the utility of MDCT in assessing early disease where luminal narrowing may not be present.

In addition to diagnosis, the presence of intimal thickening detected by IVUS also provides prognostic information regarding patient mortality and future cardiac events [31,32]. MDCT may accurately evaluate intimal thickening in CAV, yet evidence on clinical outcomes associated with disease detection is lacking. However, in native CAD, total plaque score and CAD severity as assessed by MDCT have been shown to be predictors of major adverse cardiovascular events [33]. Therefore, further study of whether early CAV detection by MDCT offers similar prognostic information is needed.

Limitations

The majority of 16-slice and 64-slice MDCT studies excluded the analysis of coronary segments smaller than 1.5 mm. This may result in underestimation of CAV, particularly in early disease where distal vasculopathy is often described. Although detection of distal disease may not result in revascularization, it still offers important diagnostic and prognostic value [34]. It may also serve as an indication for more aggressive therapy and frequent follow-up.

The complication rates associated with MDCT have not been well established, but sequelae associated with radiation and contrast exposure remain major concerns. Radiation doses for MDCT are higher than those for CCA. In studies included for analysis, effective radiation doses cited were between 3 and 10 mSv for 16-slice MDCT and between 10 and 18 mSV for 64-slice MDCT in comparison to 6 mSv for CCA [35–37]. The

National Council on Radiation Protection and Measurements identifies a risk factor for lifetime cancer mortality of 5×10^{-2} per 1 Sv exposure [38] which translates into a risk of developing a fatal cancer of 0.05–0.09% for each 64-slice MDCT and 0.03% for each CCA. The associated increased malignancy risk with repeated studies needed for detection and monitoring of CAV is an important limitation to the introduction of MDCT for disease assessment. There is an increasing number of radiation dose-reducing strategies such as ECG-controlled tube current modulation, prospective ECG-gated techniques, and image reconstruction algorithms, which were used only in one study included in this review [11]. Unfortunately, a number of these strategies depend on the presence of low heart rates which is often not feasible with transplant patients.

Along with radiation, administration of contrast required for MDCT can also lead to adverse events, most notably contrast-associated allergic reactions and contrast-induced nephropathy (CIN). The use of noniodinated contrast for radiological examinations is associated with severe allergic reactions in 0.2–0.7% of patients [38]. The incidence of renal dysfunction after administration of contrast is approximately 3% in the general patient population, but substantially higher in individuals with multiple risk factors for CIN such as transplant patients on calcineurin inhibitors [39]. Even though no reports of contrast-related adverse events were documented in our studies, use of MDCT for assessment of CAV must be balanced carefully with risk of potential allergic reaction and CIN.

Although MDCT provides an accurate assessment of the coronary arteries, it currently gives little information on left ventricular function or hemodynamics which are two important features gained by performing CCA. Impaired left ventricular function can signal underlying CAV or rejection. Similarly, the

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presence of restriction in transplant patients can similarly indicate rejection and may also portend to poor prognosis [40].

There were considerable methodological limitations with studies used in this systematic review. There was a significant selection bias, as most investigations excluded patients with clinical instability, renal dysfunction, and atrial arrhythmias. In addition, studies often did not sufficiently report characteristics that could potentially affect MDCT accuracy, such as body mass indices and the presence or absence of significant coronary calcification. Effective radiation doses and contrast-associated adverse events were also frequently not cited. In order to fully elucidate both benefit and harm of imaging, future studies must be stringent in reporting hazards associated with testing.

CONCLUSION

MDCT is a noninvasive modality that can accurately diagnose CAV when compared with CCA. Patient-based analysis noted sensitivities of 87–100% with MDCT in assessing CAV, suggesting that 8-9/10 post-cardiac transplant patients may avoid or delay screening CCA. In evaluating early CAV, MDCT also demonstrated good specificity and sensitivity when measured against IVUS. MDCT has not conclusively been shown to accurately quantify luminal narrowing associated with CAV implying that it cannot yet be used for assessment of disease progression once diagnosis has been confirmed.

ACKNOWLEDGMENTS

Abbreviations

CAV: coronary allograft vasculopathy; CIN: contrast-induced nephropathy; CCA: catheter-based coronary angiography; DSCT: dual-source computed tomography; IVUS: intravascular ultrasound; MDCT: multi-detector row computed tomography; NPV: negative predictive value; PPV: positive predictive value; QCA: quantitative coronary angiography.

Conflict of interest: none declared.

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Cardiac allograft vasculopathy: a review

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Cardiac allograft vasculopathy (CAV) is a major factor limiting long-term survival after cardiac transplantation. CAV is an accelerated form of coronary artery disease (CAD) that is characterized by concentric fibrous intimal hyperplasia along the length of coronary vessels. Both immunologic and nonimmunologic risk factors contribute to the development of CAV by causing endothelial dysfunction and injury eventually leading to progressive intimal thickening. The diagnosis of CAV remains a challenge as angiography, the standard method for detecting focal plaques, lacks sensitivity in detecting CAV, and intravascular ultrasonography, a more sensitive method, lacks the ability to evaluate the entire coronary tree. The disease is difficult to treat and results in significant morbidity and mortality. Since treatment of CAV is limited and usually involves repeat transplantation, prevention or mitigation of immunologic and nonimmunologic risk factors is critically important. CAV prevention may involve therapy that provides protection against endothelial injury implemented just before transplantation, during storage and transplantation as well as after transplantation. This review addresses the frequency of occurrence, pathophysiology, diagnosis and treatment of CAV, highlighting areas of active research.

La vasculopathie de l'allogreffon cardiaque (VAC) est un facteur important qui limite la survie à long terme après une transplantation cardiaque. La VAC est une forme accélérée de coronaropathie caractérisée par une hyperplasie fibreuse concentrique de la tunique interne des vaisseaux coronariens. Des facteurs de risque immunologiques et non immunologiques contribuent à l'apparition de la VAC en causant une dysfonction de l'endothélium et une lésion qui entraîne éventuellement l'épaississement progressif de la tunique interne. La VAC est toujours difficile à diagnostiquer, car l'angiographie, méthode normalisée de détection de la plaque focale, n'est pas assez sensible pour détecter la VAC; l'échographie intravasculaire, plus sensible, ne permet pas d'évaluer l'arbre coronarien au complet. La maladie est difficile à traiter et entraîne un taux important de morbidité et de mortalité. Comme le traitement de la VAC est limité et entraîne habituellement une nouvelle transplantation, il est crucial de prévenir ou d'atténuer les facteurs de risque immunologiques et non immunologiques. La prévention de la VAC peut faire appel à une thérapie qui protège contre les lésions endothéliales et est mise en œuvre immédiatement avant la transplantation, pendant l'entreposage et la transplantation, ainsi qu'après l'intervention. Cette critique porte sur la fréquence de l'occurrence, la pathophysiologie, le diagnostic et le traitement de la VAC et met en évidence les domaines où des recherches actives sont en cours.

Heart transplantation is the accepted therapy for patients with refractory end-stage heart disease. Although this procedure can extend and improve quality of life, it is not a cure. The median survival after heart transplantation remains 9.3 years, 11.8 years for patients surviving the first year after transplantation.[1] Cardiac allograft vasculopathy (CAV), an accelerated form of coronary artery disease (CAD), is the leading cause of death between 1 and 3 years after transplantation according to the Registry of the International Society for Heart and Lung Transplantation.[1] After year 3, CAV accounts for 17% of deaths. Angiographic studies indicate that CAV occurs in 42% of all heart transplant patients 3 years after transplantation.[1] Intravascular ultrasonography, a more sensitive technique, detects CAV in 75% of patients at 3 years. Allograft vasculopathy is a phenomenon not limited to cardiac transplantation. A similar process also limits long-term graft survival in other solid organ transplants.

CAV affects arteries, arterioles, capillaries and occasionally veins, with sparing of all recipient vessels.[2-4] The predominant feature of CAV is a diffuse, progressive thickening of the ar-

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terial intima that develops in both the epicardial and intramyocardial arteries of the transplanted heart. The process is a concentric fibrous intimal hyperplasia that appears along the entire length of the affected arteries. Included in this form of arteriosclerosis are features of both atherosclerosis and arteritis. The atherosclerotic changes range from a diffuse incorporation of lipids to the development of classic focal plaques later in the disease process. With arteritis, there is thickening of the vessel due to infiltration by mononuclear inflammatory cells responding to alloimmune or infectious stimuli. Rarely, this arteritis may progress to destroy the internal elastic lamina and involve the media.[5–8]

Differences between cardiac allograft vasculopathy and coronary artery disease

The pathological features of CAV differ significantly from those of CAD (Table 1). CAD is usually a focal, eccentric proliferation of the intima of proximal coronary vessels. There is usually sparing of the intramyocardial vessels. Fatty streaks are seen initially. Of importance in CAD is the deposition of calcium and disruption of the elastic lamina. Rarely there are signs of inflammation, and veins are never involved.

CAV is typically characterized as a diffuse concentric proliferation of the intima. Intramyocardial vessels are usually involved, and the process can even involve the coronary veins. The initial lesions seen are smooth-muscle proliferation of the intima. There is

rarely any calcium deposition,[5] the internal elastic lamina is intact and inflammation is usually present.

Pathological features

CAV is mainly a disease of the intima. Changes in the intima can be seen as early as 6 months after transplantation. The lesion at this time is a mild intimal thickening. Mild fibrosis and increases in extracellular matrix proteins may be present. Early after transplantation, intimal thickening is limited to the proximal arteries.[9] These lesions are characterized by hyperplastic fibrous thickening. Lesions then progress to a fibrofatty atheromatous plaque. Ultimately, the coronary vasculature progresses to a diffuse fibrous thickening of the intima, which can have superimposing atheromatous plaques.

The internal elastic lamina is almost always intact except for breaks that may be seen in more advanced stages of CAV. The media can be unaffected or almost completely replaced by fibrous tissue. As the intimal disease progresses in severity so does fibrosis of the media. The only vessels relatively unaffected are those with little or no muscular layer.

Pathophysiologic characteristics

The pathophysiologic features of CAV, although not completely understood, likely involve components of both immune-mediated and non-immune-mediated endothelial damage, and passenger "native vessel"

atherosclerosis.[10] There is substantial evidence that immunologic factors, including histocompatibility mismatch, acute rejection episodes and chronic inflammation, play a major role in CAV development. Nonimmunologic factors include cause of donor brain death, cytomegalovirus (CMV) infection, age, sex, obesity, dyslipidemia, hyperhomocysteinemia (HHcy), diabetes mellitus, hypertension, smoking and ischemia–reperfusion injury.[11] In general, hyperlipidemia and insulin resistance are the most significant nonimmunologic factors, occurring in 50%–80% of the heart transplant population.[12]

The endothelial damage involved in CAV can be categorized into either denuding or nondenuding injury. In nondenuding injury a rapid replacement of injured endothelial cells leads to endothelial dysfunction.[8] Both immune-related and nonimmune-related factors contribute to nondenuding injury. In contrast, denuding injury is caused by ischemia–reperfusion injury during transplantation or during episodes of acute cellular rejection. This results in the loss of large stretches of endothelium along the vessel, which causes significant endothelial dysfunction.[13] According to one hypothesis, it is the immune component or alloantigen-dependent mechanism of injury that acts principally to intensify initial nonimmune damage to the endothelial cells.[14] Denuding injury allows for blood components and circulating cytokines to have direct contact with the subintimal layers. This can lead to significant proliferation of smooth-muscle cells. Therefore, CAV can be initiated or exacerbated by several processes that can lead to denuding or nondenuding injury. These include ischemia–reperfusion injury, immune activation, viral infection and injury from immunosuppressive drugs.

Hyperlipidemia is commonly seen in cardiac transplant patients for several reasons. Many of these patients are hyperlipidemic before transplantation. In addition, the immunosup-

Table 1

Comparison between cardiac allograft vasculopathy and coronary artery disease

Characteristic	Cardiac allograft vasculopathy	Coronary artery disease
Vessel involvement	All vessel types within the allograft Mostly intramyocardial vessels	Proximal coronary vessels
Plaque pattern	Diffuse and concentric	Focal and eccentric
Inflammation	Yes	Rarely
Internal elastic lamina	Intact	Disrupted
Calcium deposition	No	Yes

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pressive therapy given to patients, especially calcineurin inhibitors, may result in or exacerbate pre-existing dyslipidemia. Hypercholesterolemia, in a rabbit heterotopic cardiac transplant model, has been shown to be associated with CAV[15,16] and transplanted coronary arteries were more affected by hypercholesterolemia than native coronary arteries. Hypercholesterolemia promotes fibrofatty proliferative changes to the intimal hyperplasia seen in most patients with CAV.[15]

In solid-organ transplant recipients, HHcy is extremely common and occurs early with a rate as high as 80%–90%.[17–28] HHcy can damage cells by several mechanisms, but primarily by affecting the endothelium.[29–31] HHcy results in reduced endothelial nitric oxide production,[32,33] impaired arterial response to vasodilators and increased expression of procoagulant factors.[22,29–31] The neutrophil–endothelium interaction is promoted in the setting of HHcy, allowing for the presence of more neutrophils in the intima. All of these alterations in the endothelial wall are caused by alterations in the redox state induced by high homocysteine levels.[30–32] Several investigators have demonstrated that HHcy is associated with the development of CAV.[34,35]

Hypertension, smoking, diabetes mellitus and other risk factors for atherosclerosis are associated with CAV. Hypertension in transplant patients can be present preoperatively or postoperatively secondary to immunosuppressive medication, such as cyclosporine. Hypertension causes endothelial injury by promoting the formation of intimal hyperplasia, which eventually gives rise to atherosclerotic lesions.

Although the relative importance of the direct versus the indirect pathway of alloreactivity is still debated, one theory is that direct activation of recipient CD4+ T cells by donor allograft/nonself major histocompatibility complex (MHC) class II molecules initiates graft rejection. CD8+ T cells can become activated by previously activated CD4+ T cells through the CD40L pathway and by nonself MHC class I molecules.

The activation of CD4+ and CD8+ T cells leads to further synthesis of cytokines, which perpetuate the ongoing cascade of events that lead to CAV. The most important cytokines in allograft rejection are interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). IL-2 induces T-cell proliferation and differentiation, IFN-γ activates macrophages, and TNF-α itself is cytotoxic to the transplanted heart. In addition, TNF-α acts to increase MHC class I expression, while IFN-γ increases the expression of both MHC classes I and II molecules. Overall, these cytokines can lead to chronic graft rejection. IFN-γ and TNF-α also induce the leukocyte vascular cell adhesion molecule-1, which promotes monocyte adhesion and entry through the endothelium, leading to CAV.

Ardehali and associates[36] used a murine CAV model with a compromised indirect alloreactivity pathway to show that this did not affect the extent of intimal thickening or lymphocyte and macrophage infiltration after heart transplantation when compared with wild type mice. They proposed 2 potential explanations: (1) an impaired indirect pathway is enough to cause severe CAV and the direct pathway does not play a major role in CAV; or (2) the direct pathway of alloreactivity can fully compensate for the impaired indirect pathway.[36] Other studies, such as the one by Game and associates,[37] have found a correlation between increased indirectly activated T cells and chronic rejection. As far as the importance of CD4+ versus CD8+ T cells, it appears that the CD4+ allorecognition pathway is required for CAV development, whereas the CD8+ pathway may act to increase the severity of CAV.[38] In a study by Szeto and colleagues,[39] hearts transplanted into CD8+ T-cell-depleted rats developed CAV, but there was no CAV in the CD4+ T-cell–depleted recipient.

These findings suggest that CAV is dependent on CD4+ indirect allorecognition and not a CD8+ direct pathway. It remains controversial as to which component of the immune response is involved in CAV, but most transplant centres agree that immune activation plays a role.

Acute rejection as a cause or risk factor for CAV has been investigated by several authors.[40–43] Some groups have reported an association between the severity and frequency of rejection and the severity of CAV; however, others have reported that episodes of acute rejection are not associated with the development of CAV.[40–44] One proposed mechanism linking acute rejection to CAV is that the inflammatory process and tissue destruction from rejection result in endothelial damage, which initiates the process of CAV or potentiates the CAV already in progress.

Recent research has correlated ischemia–reperfusion injury with CAV. Determinants of ischemia–reperfusion injury are length of ischemic time and methods of allograft storage. Gohra and associates[45] demonstrated in a rodent model of orthotopic aortic allograft transplantation that ischemia and reperfusion result in endothelial injury, leading to the development of transplant vasculopathy. They also found that endothelial cell loss occurred in both isografts and allografts due to ischemia and reperfusion.[45] This initial loss of endothelial cells was replaced by 2 weeks; however, transplant vasculopathy developed within 60 days.[45] Their study indicated that ischemia and reperfusion injury led to the development of transplant vasculopathy since isografts developed vasculopathy, although to a lesser extent than the allografts.

Several changes occur to the endothelium following hypoxia, including loss of the ability to release nitric oxide within minutes after reperfusion.[45–48] This loss is related to the consumption of nitric oxide by superoxide radicals formed early during reperfusion.[49] Ex-

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perimental evidence suggests that the oxygen free radicals are produced by neutrophils.[50] In vitro exposure of coronary arteries to oxygen radicals produces endothelial dysfunction.[50] In addition, ischemia–reperfusion causes the endothelial cells to become activated and express surface adhesion molecules. These molecules promote circulating leukocyte adhesion, which then causes endothelial damage by direct cytotoxicity.[51–53] These leukocytes become activated and release cytokines, which enhance leukocyte and smooth-muscle cell proliferation and activation. Ischemia also promotes complement activation that causes not only cell lysis, but results in several other changes such as increased vessel permeability, leukocyte chemoattraction and smooth-muscle contraction.[54,55] Complement 1-q can increase platelet procoagulant activity, which can enhance CAV by the formation of thrombus but mostly by causing the release of several vasoactive substances and growth factors such as platelet-derived growth factor-beta, thromboxane A and prostacyclin.[54] These are few of the mechanisms by which ischemia–reperfusion initiates the process of CAV. In a recent study, myocardial ischemia complicated by fibrosis in the peritransplant period was associated with increased progression of CAV and a poorer long-term outcome.[56]

Several investigators have reported an association between pathogens (*Chlamydia pneumoniae*, CMV, herpes simplex, parvovirus) and CAV. Subramanian and colleagues[57] have demonstrated that *C. pneumoniae* infection is correlated with the severity of CAV. They concluded that CAV developed in heart transplant recipients who tested positive for immunoglobulin-G against *C. pneumoniae* but not in those who tested positive for *C. pneumoniae* by polymerase chain reaction.[57] Again, this finding implicates an immunologic mechanism behind the development of CAV, regardless of the inciting stimulus. CMV infection has been as-

sociated with both atherosclerosis and CAV. The Stanford group demonstrated that severe CAV developed in approximately 30% of CMV-infected heart transplant recipients, representing a 3-fold increase compared with uninfected recipients.[58] CMV has the ability to infect vascular endothelial cells and induce endothelial injury, which can lead to CAV. Weis and colleagues[59] reported elevated asymmetric dimethylarginine (a nitric oxide synthase inhibitor) impairing vascular homeostasis in CMV-infected patients. These higher levels can lead to endothelial dysfunction and correlate with increased severity of CAV.[59] CMV and herpes simplex viruses induce the host adaptive immune response, which leads to the release of cytokines, increased expression of adhesion molecules and activation of T-cell responses. Therefore, viral infection may result in CAV by impairing nitric oxide homeostasis, inducting proinflammatory cytokines, and enhancing T-cell-mediated alloreactivity.

The incidence of significant donor CAD remains low, at approximately 2%. Donor CAD can serve as a starting point for CAV and may accelerate the disease process. Donor CAD can be important in the prognosis of the transplant patient in that it can

progress independently of the CAV process. However, Botas and associates[60] found no significant difference in the rate of intimal thickening between patients with donor hearts having pre-existing coronary artery disease and those without. Thus, the impact of native vessel atherosclerosis on CAV remains controversial.

Finally, cause of donor brain death, more specifically explosive donor brain death (v. gradual brain death), causes an up-regulation of MHC classes I and II antigens, adhesion molecules and cytokine secretion, setting off an accelerated inflammatory response in the heart.[61–63]

CAV is a complex disease with a multifactorial etiology, and several methods must be adopted to prevent its initiation and progression (Fig. 1).

Diagnosis

Cardiac denervation at the time of heart transplantation usually prevents transplant patients from experiencing angina, which is an important warning sign for heart disease. Only 10%–30% of heart transplant recipients regain any innervation to the heart. Because of this lack of early clinical symptoms, transplant patients with CAV typically present late with silent myocardial infarction, loss of
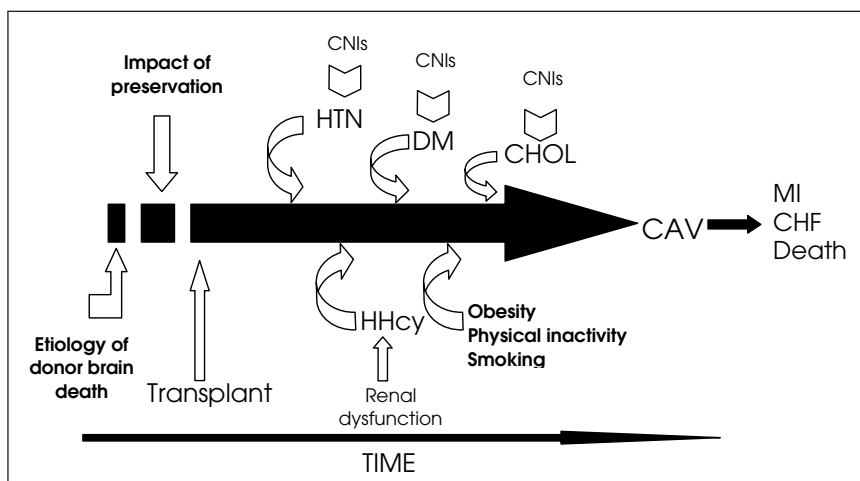
FIG. 1. Pathophysiology of cardiac allograft vasculopathy (CAV). CHF = congestive heart failure, CHOL = cholesterol, CNI = calcineurin inhibitors, DM = diabetes mellitus, HHcy = hyperhomocysteinemia, HTN = hypertension, MI = myocardial infarction.

allograft function or sudden death.[5]

Another difficulty faced by clinicians in diagnosing CAV is coronary remodelling and the diffuse nature of the disease. Angiography measures luminal diameter and compares the narrowing at plaques to normal reference diameters and previous angiograms in order to understand the severity and rate of disease progression. CAV, however, shows no initial decrease in luminal diameter due to vascular remodelling.[63] Only when the process is more advanced does the lumen narrow and angiographic detection become possible. Since CAV involves the entire coronary arterial tree, angiography may convey the impression of less-than-actual vessel narrowing at plaque sites. Thus, angiography, although it is a good screening tool for CAD, often underestimates CAV, and in some patients with evenly distributed disease throughout the coronary tree, CAV can be missed altogether.[64]

Despite the poor sensitivity of angiography, it is still widely used as a screening test for vascular disease. Johnson and associates[9] developed a classification system based on the varying morphologies in CAV to aid in its diagnosis using angiography. Briefly, type A lesions appear as discrete proximal tubular stenoses, type B as diffuse concentric middle or distal stenoses, with type B_1 having an abrupt narrowing and type B_2 having a smooth concentric tapering. Finally a type C angiographic appearance indicates irregular vessels with distal lesions and loss of small branches. Diagnosis of CAV requires type B or C lesions and comparison with previous and recent angiograms to note disease progression.[10]

A more sensitive tool is intravascular ultrasonography (IVUS). IVUS is useful for detecting the extent of intimal thickening by imaging vessel wall structure rather than simply luminal diameter. IVUS has an axial resolution of 50–80 μm.[63] Unfortunately, it is physically restricted to the larger epicardial arteries, and thus cannot be used to screen for CAV throughout the entire heart. One year after transplantation, IVUS detects CAV in 50% of patients whereas angiography detects disease in only 10%–20% of patients.[65,66]

With IVUS, normal coronary intimal thickness ranges between 0.10 and 0.30 mm. Hence, CAV is considered present when intimal thickness exceeds 0.3 mm or when the sum of the intimal and medial thickness exceeds 0.5 mm. At greater than 0.6-mm intimal thickening, patients are 10 times more likely to experience a cardiac event.[67]

Since angiography and IVUS are invasive tests, they pose increased risks for the patient.[68] Dobutamine stress echocardiography is currently the most sensitive noninvasive test for cardiac disease; it measures wall motion and can detect CAV with a sensitivity and specificity of 79% and 83% respectively.[69] Possible future modalities include both pulse-wave tissue Doppler imaging and electron-beam CT. Since both modalities are noninvasive they may replace angiography as screening tools, allowing IVUS to be used in high-risk patients or those with equivocal or positive test results.

Treatment and prevention

Treatment of established CAV in humans remains limited. Encouraging research, however, has been done in small animals. For example, treatment with anti-CD154 in a rat cardiac allograft rejection model prevents acute rejection and drastically slows the development of CAV.[70] In this study, early treatment was required to inhibit CAV.[70]

In clinical heart transplantation the focus remains on prevention of CAV via attenuation of adverse nonimmunologic and immunologic reactions. Before transplantation, preventing endothelial injury at brain death, reducing cold ischemic time and subsequent tissue damage, and improving myocardial preservation during storage and transportation of the graft all aid in post-transplant cardiac function and longevity. In a study on prolonged cold storage, Kevelaitis and associates[71] demonstrated that longer cold ischemic times produced greater endothelial dysfunction in cardiac allografts and that the composition of the storage medium affected the extent of allograft tissue damage. Our group has shown that profound hypothermic storage results in depressed myocardial metabolic and functional recovery[72] and that shed donor blood perfusion can permit cardiac allograft storage at tepid temperatures, resulting in improved myocardial performance.[72,73] We have also shown that the addition of insulin to the blood perfusate during storage results in improved functional and metabolic recovery during heart transplantation.[74] Fedak and associates[75] demonstrated that bosentan, an endothelin-1 antagonist, added to shed blood perfusion improves both the functional recovery of the myocardium and endothelium. Several other groups have demonstrated that endothelin antagonism reduces CAV.[76,77]

Immediately after transplantation, patients are placed on calcineurin immunosuppressive drugs (cyclosporine or tacrolimus), most commonly cyclosporine. Unfortunately, cyclosporine in high doses and for a long time can cause side effects such as renal failure[4] and hypertension. Simonson and colleagues[78] demonstrated in a Lewis to Fischer rat heart transplant model that the combination of low-dose cyclosporine with an endothelin-converting enzyme inhibitor resulted in long-term survival of the graft equal to that of high-dose cyclosporine alone. As an alternative to using cyclosporine, other immunosuppressive drugs such as mycophenolate mofetil, rapamycin or leflunomide, may inhibit CAV by limiting smooth-muscle cell proliferation.[4] The newer immunosuppressive agent everolimus has recently been demon-

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strated to reduce the frequency and severity of CAV[79] in humans. Eisen and associates[79] demonstrated that, in patients on cyclosporine and corticosteroids, everolimus reduces intimal thickness and index compared with azathioprine.

Hyperlipidemia is known to be a risk factor for both CAD and CAV. Unfortunately, immunosuppressive therapy with corticosteroids, cyclosporine, rapamycin and to a lesser extent tacrolimus and everolimus results in hyperlipidemia.[80] To treat hyperlipidemia in post-transplant patients, lipid-lowering drugs are prescribed since lifestyle changes are usually not enough to lower lipid profiles to desired levels. HMG-CoA reductase inhibitors, or statins, are the most popular and are very effective at lowering total cholesterol, low-density lipoprotein (LDL) and very low-density lipoprotein, and increasing high-density lipoprotein (HDL). Recently, it has been documented that statins have pleiotropic effects in that they improve vascular function. Statins decrease endothelial dysfunction through increasing nitric oxide production, inhibiting the coagulation cascade and limiting oxidized-LDL-mediated damage to the endothelium.[81–83] Pravastatin is the most commonly used HMG-CoA reductase inhibitor after heart transplantation. In 1995, Kobashigawa and associates[84] showed that treatment with pravastatin (40 mg/d) for 1 year, lowered mean LDL and triglyceride levels, raised HDL levels and reduced intimal thickening and cardiac rejection accompanied by hemodynamic compromise ($p = 0.002$) (Fig. 2). In this trial, patients treated with pravastatin had a lower incidence of CAV and improved survival ($p = 0.025$)[84] (Fig. 3). These effects may be enhanced through immunosuppression modulation since a subgroup of patients on pravastatin had significantly reduced cytotoxicity of natural killer cells.[84] Simvastatin, likewise, has beneficial lipid-lowering effects in heart transplant recipients.[85] In addition,

Simvastatin inhibits proliferation and induces apoptosis of vascular smooth-muscle cells.[86] However, simvastatin has a low but significant rate of rhabdomyolysis and myositis. Thus, Keogh and associates[85] proposed that pravastatin be the statin of choice in heart transplantation. Atorvastatin has been shown to further reduce LDL in heart transplant recipients who are resistant to pravastatin or simvastatin. However, of the 48 patients who had received a mean (and standard deviation) atorvastatin dose of 21 (10) mg, 2 suffered from myositis, and myalgias appeared in another 2 patients. The study concluded that the drug was safe in moderate doses with careful patient monitoring.[87]

HHcy in the cardiac transplant patient affects long-term outcomes by leading to the development of CAV. Several investigators have demonstrated that folic acid and vitamin B_{12} supplementation can significantly reduce homocysteine levels in the cardiac transplant patient.[88–90] Kutschka and associates[90] demonstrated that folic acid supplementation (5 mg/d) can effectively lower elevated homocysteine levels in heart transplant recipients. Unfortunately, these studies revealed only that homocysteine levels

FIG. 2. Maximal intimal thickness 1 year after cardiac transplantation. Pravastatin significantly attenuated intimal proliferation during the first year after transplantation. White = baseline, grey = increase from baseline to 1 year. Reproduced with permission from Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, et al. Effect of pravastatin in outcomes after cardiac transplantation. *N Engl J Med* 1995;333:621-7.

can be lowered and did not demonstrate if reduction leads to decreased severity or prevalence of CAV.

There is general acceptance that alloimmunity plays a role in CAV. The occurrence of CAV increases as the number of HLA mismatches increases.[91,92] Before transplantation most patients have a panel reactive antibody (PRA) test performed. A PRA result greater than 10% is considered positive and indicates that the recipient will be at higher risk for graft rejection. Kerman and associates[93] demonstrated that recipients with PRAs greater than 10% had a 2-fold increased risk for CAV. Immune modulation to lower PRAs has the potential to reduce acute rejection and may limit the development of CAV. Treatment strategies to lower PRAs include the intravenous use of immunoglobulin, plasmapheresis, cyclophosphamide, mycophenylate mofetil and azathioprine. The optimal strategy to prevent alloimmune injury would be to induce tolerance. Host tolerance to the allograft will abolish rejection and the immune component of CAV development. Although not achieved clinically, several investigators have demonstrated that tolerance can be induced in experimental models.[94–97]

Once CAV has been established,

FIG. 3. Survival during the first year after cardiac transplantation. Treatment with pravastatin significantly ($p = 0.025$) improved survival compared with controls. Solid line = control, dotted line = pravastatin. Reproduced with permission from Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, et al. Effect of pravastatin in outcomes after cardiac transplantation. *N Engl J Med* 1995;333:621-7.

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treatments such as coronary angioplasty, coronary stenting, and coronary bypass offer only palliative solutions. The only true solution to severe CAV is repeat heart transplantation. Even so, CAV is likely to recur, and there are significant moral and ethical issues that complicate repeat transplantation.[5,98]

Summary and conclusions

CAV is the major limiting factor for long-term survival after heart transplantation. It affects up to 75% of patients 3 years after transplantation. The risk factors for CAV can be divided in 2 categories (immunologic and nonimmunologic). Immunologic factors include the severity and frequency of acute rejection, and chronic rejection. Nonimmune factors include the classic risk factors for CAD, ischemia–reperfusion injury during organ retrieval and transplantation, CMV infection and endothelial injury from immunosuppressive drug therapy. Current areas of research focus on determining the etiology of CAV and the development of treatment strategies to prevent or limit its extent. These include endothelial protection during organ retrieval, limiting the use of calcineurin inhibitors and aggressive management of CAD risk factors. Another challenge in the management of CAV is its diagnosis. Early diagnosis of CAV will lead to earlier treatment and better outcomes. Angiography — the standard diagnostic modality for CAD — lacks sensitivity, and IVUS (the most sensitive method) lacks the ability to assess the entire coronary tree. New diagnostic tools are required for the more accurate and earlier diagnosis of CAV. The successful long-term survival of the cardiac transplant patient rests in our ability to understand, detect, treat and prevent CAV.

CAV is a multifactorial disease that remains the major limitation to long-term survival after heart transplantation. Methods of diagnosis have improved significantly with the use of IVUS in addition to angiography. Since treatment of CAV is limited and usually involves repeat transplantation, prevention of immunologic and nonimmunologic risk factors is of critical importance. CAV is conceptually very similar to post-transplant disorders in other organs (e.g., bronchiolitis obliterans with organizing pneumonia, biliary cirrhosis). Therefore, novel therapeutic strategies to prevent or attenuate the development of CAV may have clinical relevance to transplant recipients of other solid organs.

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iREVIEWS

STATE-OF-THE-ART PAPER

Detection and Imaging of Cardiac Allograft Vasculopathy

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Cardiac allograft vasculopathy (CAV) is an important cause of morbidity and mortality among cardiac transplant recipients. CAV occurs in approximately 30% of patients by 5 years and 50% by 10 years, and is a major cause of graft loss and death. Early detection of CAV is important because it may allow alterations in medical therapy before progression to the stage that revascularization is required. This has led to routine screening for CAV in transplant recipients, traditionally by invasive coronary angiography (ICA). Recent advances in imaging technology, specifically intravascular ultrasound, now also permit detection of subangiographic CAV. Noninvasive stress testing and multislice coronary computed tomography angiography have been investigated as noninvasive alternatives to routine ICA. However, currently available noninvasive tests remain limited with respect to their sensitivity and specificity for CAV. Given the multiple available diagnostic modalities, no consensus definition for the classification of CAV has been widely accepted, although new guidelines that rely heavily on ICA have recently been published by the International Society of Heart and Lung Transplantation. This review summarizes imaging modalities that are utilized in the diagnosis and surveillance of CAV and explores newer imaging techniques that may play a future role.   (J Am Coll Cardiol Img 2013;6:613–23) © 2013 by the American College of Cardiology Foundation

Cardiac transplantation is the definitive treatment for end-stage cardiomyopathy of any cause. The International Society for Heart and Lung Transplantation estimates that more than 5,000 heart transplants are performed each year worldwide (1). In 2010, 2,333 transplants were done in the United States alone (2). The median survival after transplantation has steadily improved and now exceeds 10 years (1). With improvement in long-term survival, cardiac allograft vasculopathy (CAV) has become an increasingly important cause of morbidity and mortality among transplant recipients (3). The incidence of CAV is approximately 8% at 1 year, 30% at 5 years, and 50% at 10 years, and it is a major cause of graft loss and death after the first year (1).

CAV is an accelerated fibroproliferative process that affects the coronary arteries of cardiac allografts (4). Risk factors include the number of HLA-DR mismatches; older donor age and

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donor history of hypertension; and younger recipient age, and presence of recipient diabetes and obesity (1,5). The pathophysiology of CAV involves immunologic and nonimmunologic factors that cause inflammation with persistent vascular injury and endothelial dysfunction (6–9). Histologically, there is subendothelial accumulation of lymphocytes (primarily T cells), myointimal proliferation of smooth muscles cells, development of lipid-laden foam cells, and perivascular fibrosis (10–12). Concentric intimal hyperplasia leads to progressive luminal compromise resulting in a diffuse obliterative process of the intramural and epicardial coronary arteries (Fig. 1) (13–17). Eventually, decreased coronary blood flow and reduced vasodilatory capacity lead to ischemia and ventricular dysfunction

(18,19). By contrast, traditional atherosclerotic coronary artery disease (CAD) typically evolves over a longer time period, is focal and noncircumferential, and involves more calcium deposition during the late stages (Fig. 2) (4). Although CAV typically manifests as diffuse luminal narrowing, lesions can also be focal and eccentric with an appearance similar to typical CAD (16,17,20). Since CAV can occur in combination with CAD, which may be present in allografts due to either de novo atherosclerosis or pre-existing donor atherosclerosis (20–22), distinguishing between these processes may be challenging. It remains to be determined whether this distinction will be important from a clinical standpoint.

The early detection of CAV is critically important because it may allow alterations of medical therapy before progression to the stage that revascularization is required. There is evidence that modification of immunosuppressive regimens may delay CAV progression or even cause regression (23–26). However, early diagnosis remains challenging due to absent or atypical symptomatology related to allograft denervation (27–31). Although cardiac reinnervation may occur in 10% to 30% of cases, typical angina remains uncommon (27,31). In 1 study of 29 myocardial infarctions identified in explanted allografts at autopsy or retransplantation, a history of chest or arm pain was reported in only 12% of cases (30). In fact, the initial manifestations of CAV can be allograft dysfunction, silent myocardial infarction, or sudden death (29). This has led to routine screening of heart transplant recipients for CAV, most often

with invasive coronary angiography (ICA). Although CAV typically progresses gradually, it can also evolve rapidly and unpredictably (19,32–34). Rapid progression of CAV, especially in the first 5 years after transplantation, is a powerful predictor of the development of advanced disease, myocardial infarction, and mortality (35–37).

CAV was initially diagnosed by histopathologic examination, but advances in management have prolonged graft and patient survival, allowing for angiographic diagnosis. Advances in imaging technology, specifically intravascular ultrasound (IVUS), now also permit detection of subangiographic CAV. Noninvasive stress testing, particularly with dobutamine stress echocardiography (DSE), has been investigated as a way to decrease the need for ICA. Recently, multislice coronary computed tomography angiography (CTA) has also been investigated as a noninvasive alternative to ICA. However, these noninvasive tests remain limited with respect to their sensitivity and specificity for the diagnosis of CAV. In 2010, the International Society for Heart and Lung Transplantation published a new consensus document for the classification of CAV based primarily on angiography (3). The purpose of this review is to describe the imaging modalities that are utilized in the diagnosis and prognostic surveillance of CAV.

Diagnosis of CAV

Noninvasive stress testing. Due to the high frequency of baseline electrocardiogram abnormalities and the reduced exercise capacity of cardiac transplant recipients, the specificity of exercise stress

Figure 1. Histology of CAV

Marked concentric intimal hyperplasia and proliferation associated with cardiac allograft vasculopathy (CAV) along with a predominance of lymphocytes and foam cells. Image courtesy of Dr. Charles Marboe, Columbia University Medical Center.
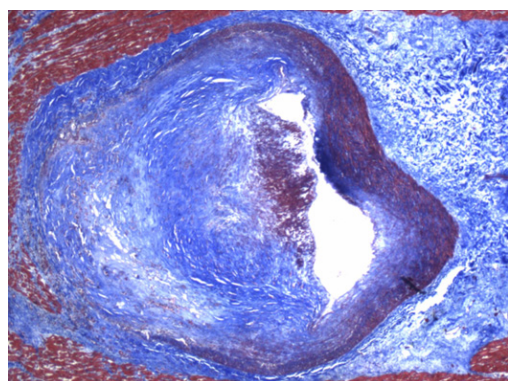
Figure 2. Histology of Native Coronary Atherosclerosis

An example of a primarily fibrotic plaque with a paucity of lymphocytes. Notice the eccentric proliferation of the intima, in contrast to the concentric intimal hyperplasia commonly seen in cardiac allograft vasculopathy (CAV). Image courtesy of Dr. Charles Marboe, Columbia University Medical Center.

electrocardiography alone for detection of CAV is poor and adjunctive imaging is generally required (38). Echocardiography and radionuclide imaging have been used to improve both sensitivity and specificity.

Multiple studies have compared the performance of DSE and ICA with or without IVUS for detecting the presence and severity of CAV (39–44). Studies have demonstrated that regional myocardial dysfunction as assessed by DSE correlates well with IVUS evidence of moderate to severe intimal hyperplasia in cardiac allografts (42,43). In comparison to ICA, the sensitivity, specificity, positive predictive value (PPV),

and negative predictive value (NPV) of DSE for the diagnosis of CAV range from 65% to 95%, 55% to 95%, 69% to 92%, and 71% to 92%, respectively (Table 1) (39,41,44). The relatively strong NPV of DSE compared with ICA has led some centers to use DSE to increase the interval between invasive screening tests in the appropriate clinical setting. With the addition of IVUS to ICA, the sensitivity, specificity, PPV, and NPV of DSE for CAV range from 72% to 79%, 83% to 88%, 88% to 92%, and 62% to 71%, respectively (Table 1) (42,43). The lower sensitivity and NPV of DSE compared with IVUS is most likely a reflection of the superior sensitivity of IVUS relative to ICA for detection of CAV (see the following text).

Assessment of coronary flow reserve with dobutamine stress contrast-enhanced transthoracic echocardiography is in the preliminary stages of investigation as a screening test for CAV (45–47). In 1 study, this modality was compared with ICA in 35 asymptomatic transplant recipients and had a sensitivity of 70% and specificity of 96% for the presence of a focal stenosis >50% (47). However, abnormalities were detected in only 1 of the 5 patients with multivessel disease. This method requires high image quality, which may be difficult to achieve in some cardiac transplant recipients due to suboptimal acoustic windows.

Stress radionuclide myocardial perfusion imaging (MPI), with either exercise or pharmacological stress modalities, has also been evaluated for the detection of CAV (48–51). The sensitivity of exercise stress MPI for detection of traditional CAD has been shown to be decreased in patients with

Table 1. Stress Tests in the Evaluation of CAV

First Author (Ref. #)	Modality	Patients (N)	Comparator	CAV Diagnosis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Derumeaux et al. (39)	DSE	37	ICA	Angiographic focal stenosis >50%	65	95	92	76
Akosah et al. (40)	DSE	41	ICA	Angiographic luminal irregularities or diffuse disease	95	55	69	92
Spes et al. (42)	DSE	98	ICA or IVUS	Angiographic luminal irregularities or IVUS MIT >0.3 mm	72	88	92	62
Spes et al. (43)	DSE	46	IVUS	MIT >0.3 mm	79	83	88	71
Derumeaux et al. (44)	DSE	41	ICA	Angiographic luminal irregularities or diffuse disease	86	91	86	91
Ciliberto et al. (49)	Dipyridamole technetium-99m sestamibi SPECT	78	ICA	Angiographic focal stenosis >50% or diffuse disease	92	86	55	98
Ciliberto et al. (49)	Dipyridamole technetium-99m sestamibi SPECT	78	ICA	Angiographic luminal narrowing (any CAV)	56	89	70	81
Wu et al. (50)	Dobutamine thallium SPECT	47	ICA	Angiographic focal stenosis >50% or diffuse disease	89	71	42	96
Elhendy et al. (51)	Dobutamine technetium-99m tetrofosmin SPECT	50	ICA	Angiographic focal stenosis >50%	90	55	75	79

CAV = cardiac allograft vasculopathy; DSE = dobutamine stress echocardiography; ICA = invasive coronary angiography; IVUS = intravascular ultrasound; MIT = maximal intimal thickness; SPECT = single-photon emission computed tomography; NPV = negative predictive value; PPV = positive predictive value.

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submaximal exercise tolerance (52–54). The limited sensitivity of this modality for detection of CAV, ranging from 60% to 77% in available studies, may therefore be related to the known impairment of exercise tolerance in cardiac transplant recipients (48,55–57). The use of pharmacological stress agents has been demonstrated to improve the sensitivity and NPV, but the specificity and PPV remain limited (Table 1). Furthermore, the diagnostic performance of stress MPI has been shown to be even poorer for the detection of nonobstructive (<50% stenosis) CAD (49).

Overall, noninvasive stress testing modalities, specifically DSE and pharmacological MPI, offer reasonable sensitivity and NPV for the detection of significant obstructive CAV. However, these modalities have limited specificity and PPV for CAV, and remain insufficiently sensitive to reliably detect early-stage or limited disease. Thus, invasive techniques remain the standard of care for the diagnosis and surveillance of CAV.

Coronary angiography. Invasive coronary angiography is the screening and surveillance test of choice for CAV at most cardiac transplant centers and is typically performed on a routine periodic basis (3). The prevalence of CAV detected by ICA is approximately 10% to 20% at 1 year and 35% to 50% at 5 years (9,34,58–60). The angiographic diagnosis of CAV has prognostic significance for graft survival, patient survival, and adverse cardiac events. However, concern remains regarding the sensitivity of coronary angiography for CAV when compared with IVUS and histopathologic studies (3,61–64). Coronary angiography also exposes patients to the risks of an invasive procedure and necessitates the use of iodinated contrast, which may pose an increased risk of kidney injury in cardiac transplant recipients, among whom chronic kidney disease is a common comorbidity (65).

Multiple definitions and classification schemes for the angiographic diagnosis of CAV exist in the literature, with stenosis thresholds ranging from 30% to 70% (9,62,66). Early work by Gao and colleagues (66) attempted to standardize the description of CAV lesion type and led to a classification system that was widely adopted. However, the classification system subsequently failed to demonstrate prognostic significance, leading to the development of newer classification schemes (3). New guidelines for the classification of CAV were published in 2010 by the International Society of Heart and Lung Transplantation (3). They integrate cor-

Table 2. ISHLT Recommended Nomenclature For CAV

Classification	Severity	Definition
ISHLT CAV_0	Not significant	No detectable angiographic lesions
ISHLT CAV_1	Mild	Angiographic LM <50%, or primary vessel with maximum lesion of <70%, or any branch stenosis of <70% (including diffuse narrowing) without allograft dysfunction
ISHLT CAV_2	Moderate	Angiographic LM ≥50%; a single primary vessel ≥70%, or isolated branch stenosis of ≥70% in branches of 2 systems, without allograft dysfunction
ISHLT CAV_3	Severe	Angiographic LM ≥50%, or 2 or more primary vessels ≥70% stenosis, or isolated branch stenosis ≥70% in all 3 systems; or ISHLT CAV_1 or CAV_2 with allograft dysfunction (defined as LVEF ≤45%, usually in the presence of regional wall motion abnormalities) or evidence of significant restrictive physiology

A "primary vessel" denotes the proximal and middle 33% of the left anterior descending artery, the left circumflex, the ramus, and the dominant or codominant right coronary artery with the posterior descending and posterolateral branches. A "secondary branch vessel" includes the distal 33% of the primary vessels or any segment within a large septal perforator, diagonals, and obtuse marginal branches or any portion of a nondominant right coronary artery. Restrictive cardiac allograft physiology is defined as symptomatic cardiac allograft physiology is defined as symptomatic heart failure with echocardiographic E to A velocity ratio of >2 (>1.5 in children), shortened isovolumetric relaxation time (<60 ms), shortened deceleration time (<150 ms), or restrictive hemodynamic values (right atrial pressure >12 mm Hg, pulmonary capillary wedge pressure >25 mm Hg, cardiac index <2 l/min/m^2). Adapted from the 2010 ISHLT consensus statement for recommended nomenclature of CAV (3).
 CAV = cardiac allograft vasculopathy; ISHLT = International Society of Heart and Lung Transplantation; LM = left main coronary artery; LVEF = left ventricular ejection fraction.

onary angiographic findings with assessment of graft function and hemodynamics (Table 2).

Although ICA remains the standard of care, studies have raised concern regarding its limited sensitivity for the detection of early-stage CAV (62,67–69). ICA assesses the vessel lumen, but does not permit evaluation of the arterial wall. Although traditional CAD typically causes focal, eccentric narrowing of the vessel lumen, CAV is more often a diffuse process that initially manifests as intimal thickening and evolves into concentric, longitudinal lesions (Fig. 3) (13–16,70). This process may be difficult to detect by luminal assessment alone, particularly in the early stages. In a histopathologic study of 10 explanted allografts within 2 months of a normal ICA, 75% were found to have significant intimal hyperplasia, indicative of CAV (61). Studies comparing ICA with IVUS have demonstrated sensitivity, specificity, PPV, and NPV for the diagnosis of CAV that range from 43% to 44%, 81% to

95%, 90% to 92%, and 27% to 57%, respectively (63,71). The limited sensitivity and NPV of ICA relative to IVUS has led to increasing interest in the use of intravascular imaging to facilitate the early diagnosis of CAV.

Intravascular imaging. The initial manifestations of CAV are confined to the arterial wall, so the ability of intravascular ultrasound to define the intimal and medial layers of the coronary arteries makes it particularly useful in the assessment of early-stage disease (Fig. 4). On IVUS assessment, half of transplant recipients with angiographically normal coronary arteries later than 1 year after transplantation have moderate or severe intimal thickening of the left anterior descending coronary artery (69). Due to its superior sensitivity, IVUS is now being used by some centers as the diagnostic test of choice for detection of early CAV.

The use of IVUS has also led to important advances in the understanding of the natural history, distribution, and morphology of CAV (16,17,20–22,72,73). Serial IVUS imaging has shown that the dynamic process of coronary artery remodeling begins within the first year after transplantation (74). Initially, there is thickening of the coronary intima, which occurs in more than 80% of patients within 1 year after transplantation (16,17,63). In addition, affected arteries develop an early expansion of the external elastic membrane with preservation of luminal area (75–77). Changes in the external elastic membrane tend to occur in a biphasic pattern, with early expansion followed by late, concentric remodeling and reductions in luminal area (77).

Radiofrequency IVUS has been investigated in traditional atherosclerotic CAD to delineate the components of coronary lesions and classify plaque based on its composition (78–80). In transplant recipients, it has demonstrated that lesions in the early post-transplant period tend to be focal, noncircumferential, and composed mainly of fibrous and fibrofatty tissue. The presence of lesions with a necrotic core early after transplantation is associated with older donors, male recipients, and diabetes and may represent traditional atherosclerosis rather than CAV (22,72). Fibrous and fibrofatty plaque remain the predominant lesions, regardless of time from transplantation, and can be representative of either CAV or traditional atherosclerosis (11,13,22). However, calcified lesions with necrotic cores, markers of traditional atherosclerotic disease, begin to increase within 2 years of
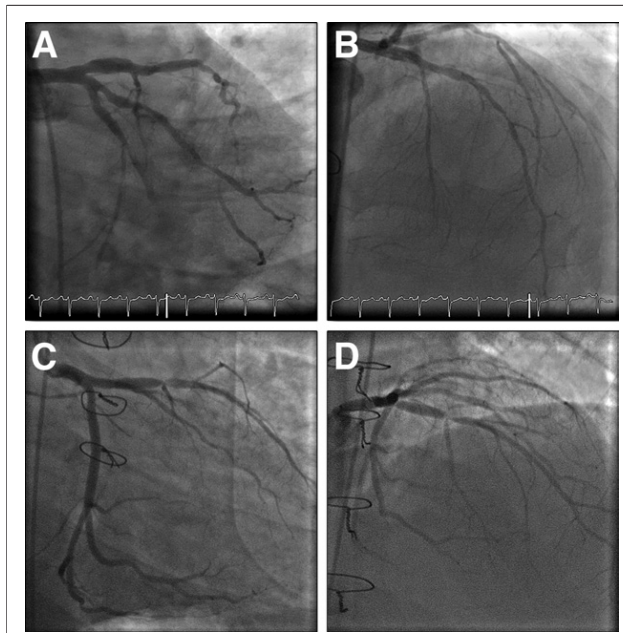
Figure 3. Distinct Manifestations of CAV

Coronary angiography demonstrating distinct manifestations of cardiac allograft vasculopathy. **(A and B)** The classical angiographic appearance of cardiac allograft vasculopathy (CAV) with multiple sequential lesions, diffuse narrowing of the coronary arteries, and prominent pruning of the distal vasculature. **(C and D)** CAV can also appear similar to typical atherosclerotic coronary artery disease in a native heart. A focal, proximal left anterior descending (LAD) coronary artery lesion.

transplantation and become more prevalent with time (72). Inflammatory plaques (composed of >30% necrotic core with dense calcification) are associated with a higher rejection score as well as subsequent progression of CAV when compared with noninflammatory plaques (26). The clinical use and interpretation of radiofrequency IVUS must be undertaken with caution since it has not been well validated by histopathologic studies of cardiac allografts.

Despite its superior sensitivity and the demonstrated prognostic value of intimal thickening, IVUS has not been widely adopted as a routine screening test for CAV due to several limitations. Intimal proliferation assessed by IVUS does not necessarily correlate with small-artery disease by histologic or immunohistochemical analysis (3,81). In addition, the size of currently available IVUS catheters makes direct imaging of the distal, small-caliber vasculature technically difficult (82). Furthermore, multivessel imaging is necessary to optimize the sensitivity of IVUS for detection of CAV. In 1 study, the prevalence of CAV in patients with 1, 2, or 3 vessels imaged

Figure 4. IVUS Imaging of CAV

Intravascular ultrasound (IVUS) frames of transplant vasculopathy corresponding to **(A)** middle left anterior descending artery (LAD), **(B)** proximal LAD, and **(C)** distal left main (LM) of the same patient after 1 year of heart transplantation. The major morphological plaque characteristics are the diffuse atheromatosis, the concentric, rather than eccentric, disease pattern, the lack of significant calcification, and an integration of positive and negative remodeling. CAV = cardiac allograft vasculopathy. Images courtesy of Dr. Elias Sanidas, Cardio-vascular Research Foundation.

was 27%, 41%, and 58% at 1 year and 29%, 55%, and 74% at 3 years (83). The complication rate of multivessel IVUS imaging has been shown to be 1.6% (84). Finally, increased cost and lack of expertise may limit the widespread availability of IVUS. For these reasons, the routine use of IVUS for the detection and surveillance of CAV is not currently recommended.

Since the pathophysiology of CAV and conventional atherosclerosis are distinct, an imaging modality with the capability for near-histologic characterization may be potentially useful from a diagnostic, prognostic, and therapeutic perspective. Optical coherence tomography (OCT) is a high-resolution (10 μm), intravascular, light-based imaging modality that measures the intensity of reflected light waves and converts these echoes into a high-resolution tomographic image (85). OCT has been used to visualize and characterize coronary atherosclerotic plaque composition, including intimal hyperplasia, delineate between intima and plaque, resolve thin fibrous caps, image beyond calcified tissue, and differentiate between fibrous and lipid-rich plaque (86,87). The potential role of OCT imaging in cardiac transplant recipients is currently being studied.

Cardiac computed tomography angiography. Cardiac computed tomography angiography has the potential to be a noninvasive alternative to ICA for the diagnosis of CAV. In addition to the vessel lumen, coronary CTA also allows some evaluation of the arterial wall, which could potentially enhance its sensitivity for CAV. Modern multidetector and dual-source technology has significantly improved spatial and temporal resolution, allowing adequate

evaluation of 81% to 100% of coronary segments >1.5 mm in diameter (88–95).

Several studies have compared the performance of multidetector (16- or 64-slice) and dual-source coronary CTA with ICA and/or IVUS for the diagnosis of CAV (Table 3). The consistently high NPV of coronary CTA relative to ICA has led to interest in its use as the primary screening test for CAV. A potential future benefit of coronary CTA is the ability to evaluate wall thickening that is not apparent by ICA. However, with current technology, the NPV of coronary CTA as compared with IVUS remains low, reflecting the superior sensitivity of IVUS.

Important limitations remain with respect to the use of coronary CTA in the evaluation of CAV. Existing studies have limited analysis to coronary segments >1.5 mm in diameter, and evaluation of the smaller distal vasculature, where CAV often manifests, may be inadequate. Chronic kidney disease is a common comorbidity in cardiac transplant recipients, and the risk of kidney injury may be increased with coronary CTA, which typically requires a large contrast load. In fact, the majority of trials of coronary CTA for CAV have excluded patients with significant renal insufficiency (90,91,96–99). The radiation dose with coronary CTA is also typically higher than with standard ICA, and this may be of particular concern in cardiac transplant recipients who require serial radiographic examinations (91,92,97,98). Many of the limitations of coronary CTA are likely to improve with ongoing advances in imaging technology. Very limited data are available regarding the

prognosis of cardiac transplant recipients followed by serial coronary CTA.

Prognosis of CAV

Noninvasive stress testing. Multiple studies have demonstrated the prognostic significant of non-invasive stress testing in cardiac transplant recipients. Studies have shown that a normal DSE in this setting has a NPV ranging from 92% to 100% for subsequent cardiac events (41,42,100). This strong NPV has led some centers to use DSE to increase the interval between required ICA in appropriate clinical circumstances, particularly when serial angiograms are normal. On the other hand, studies have demonstrated that abnormal myocardial perfusion as assessed by stress MPI is an independent predictor of cardiac death in transplant recipients (50,93,101). Furthermore, both abnormal and serially worsening DSE findings have been shown to predict major cardiac events in transplant recipients (42). Overall, the current literature supports a potential role for noninvasive stress testing to lengthen the interval between required ICA or as a screening test for CAV in patients with a contraindication to ICA (i.e., renal failure or contrast allergy).

Coronary angiography. The diagnosis of CAV by ICA has also been shown in multiple studies to have prognostic importance in cardiac transplant recipients. In 1 study, the lack of significant angiographic CAV was a significant predictor of survival without adverse cardiac events at 2 years (88). In another study of nearly 6,000 post-transplant angiograms, the 5-year rate of death or retransplantation as a result of CAV was 7% among all patients and 50% among patients with severe CAV (9). Both the overall burden and the rate of progression of CAV have also been shown to be prognostically important. In 1 study, patients with single-vessel vessel disease had survival of 64% at 1 year and 36% at 2 years, but patients with triple-vessel CAV had a much worse prognosis with a survival of only 13% at 2 years (102). In another study, transplant recipients with moderate, single-vessel disease who did not demonstrate angiographic progression at 1 year were likely to remain free of lesion progression and adverse clinical events for up to 6 years (94). However, the approximately 50% of patients who were found to have progression of CAV at 1 year required higher rates of revascularization and had significantly increased risk of sudden death (58,94). The demonstrated prognostic significance of the presence, burden, and progression of CAV as assessed by ICA is one of the reasons that it remains the current standard of care for the diagnosis and surveillance of CAV.

Intravascular imaging. The IVUS diagnosis of CAV presence and progression has also been shown to predict adverse cardiac events and death in cardiac transplant recipients (16,36,37,95). In 1 study, severe intimal thickening, defined as maximal intimal thickness (MIT) >0.5 mm, conferred a 7-fold increased risk of major cardiac events (35). Rapidly progressive CAV, defined as an increase of 0.5 mm in MIT at a given coronary site within the first year after cardiac transplantation, is a particularly poor prognostic indicator that has been associated with subsequent devel-

Table 3. Coronary Computed Tomographic Angiography for the Diagnosis of CAV

First Author (Ref. #)	Modality	Patients/ Coronary Segments	Evaluable Segments	Comparator	CAV Diagnosis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Sigurdsson et al. (91)	MDCT-16	54/791	754 (95)	ICA	Angiographic stenosis >50%	86	99	81	99
Sigurdsson et al. (91)	MDCT-16	13/154	154 (100)	IVUS*	MIT >0.5 mm	96	88	80	98
Schepis et al. (92)	DSCT-64	30/459	441 (96)	ICA	Angiographic stenosis >50%	93	80	48	98
Schepis et al. (92)	DSCT-64	30/114	110 (97)	IVUS	MIT >0.5 mm	85	84	76	91
Pichler et al. (96)	MDCT-16	60/757	711 (94)	ICA	Angiographic stenosis >50%	71	>99	91	99
Romeo et al. (98)	MDCT-16	50/450	432 (96)	ICA	Angiographic stenosis >50%	80	>99	80	>99
von Ziegler et al. (99)	MDCT-64	26/371	302 (81)	ICA	Angiographic stenosis >50%	88	97	48	100
Gregory et al. (97)	MDCT-64	20/122	119 (98)	IVUS	MIT >0.5 mm	70	92	89	77

Values are n/N or n (%). Sensitivity, specificity, PPV, and NPV are reported as per segment, after excluding non-evaluable segments. *Qualitative assessment only of proliferative changes after excluding segments with significant stenosis by quantitative coronary angiography.
DSCT = slice dual-source computed tomography; MDCT = slice multidetector computed tomography; other abbreviations as in Table 1.

opment of severe CAV, myocardial infarction, graft loss, and mortality (16,36,37). This suggests that the presence and progression of intimal proliferation, as assessed by IVUS measurement of MIT, may be a useful prognostic test.

Proposed diagnostic algorithm. An evidence-based screening algorithm for CAV can be proposed based on the literature detailed in the preceding text. Routine periodic screening for CAV is necessary due to absent or atypical symptomatology in the majority of cardiac transplant recipients. It is essential that every patient undergo ICA 1 year after cardiac transplantation. An early angiogram (with or without IVUS) 1 month after transplantation may be considered to establish a baseline assessment. Abnormal findings at that time are unlikely to represent CAV and more likely reflect donor CAD. After the first annual study, ICA is recommended on at least a biennial basis in patients without CAV and on an annual basis in those with CAV. Centers with intravascular imaging expertise may also elect to perform IVUS at the time of ICA to identify early-stage, subangiographic CAV. In patients who are found to have normal coronaries on serial ICA, the surveillance interval may be increased, potentially with the use of noninvasive stress testing, such as DSE. In general, noninvasive stress testing, specifically DSE and MPI, is insufficiently sensitive to detect early disease, and is reserved for this purpose or for patients with contraindications to ICA.

Future Directions

Although ICA remains the standard of care for the diagnosis and surveillance of CAV, IVUS studies have demonstrated the potential incremental value of modalities that allow assessment of the arterial wall in addition to the vessel lumen. With continued technological advances, improvement in the ability of coronary CTA to assess the arterial wall and distal small vessels, while requiring less contrast and radiation, may allow it to play an increasing role in this arena. The development of specific pharmacological therapy for CAV may also increase the clinical urgency of developing techniques to detect very early disease and distinguish lesion types. Given the high resolution of OCT, it may allow for earlier diagnosis as well as detailed analysis of CAV lesion types, Further research will be required to determine whether novel imaging techniques for CAV will alter the management and clinical outcomes of cardiac transplant recipients. Given the limited patient population, researchers will need to collaborate through multicenter studies to advance this important and growing field.

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Key Words: angiography ▪ cardiac allograft vasculopathy ▪ intravascular ultrasound ▪ OCT ▪ transplant.

ARTICLE

Occult and Frequent Transmission of Atherosclerotic Coronary Disease With Cardiac Transplantation

Insights From Intravascular Ultrasound

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ABSTRACT: *Background* Transplant coronary artery disease is a major cause of morbidity and mortality after cardiac transplantation. However, limited data exist regarding the potential contribution of coronary atherosclerosis in the donor heart to cardiac-allograft vasculopathy. *Methods and Results* We performed quantitative coronary angiography and intravascular ultrasound imaging in 50 of 62 consecutive heart-transplant recipients (40 men, 10 women, mean age, 53±9 years) 4.6±2.6 weeks after transplantation. The donor population consisted of 30 men and 20 women (mean age, 32±12 years). Ultrasound imaging visualized all three coronary arteries in 22 patients, two coronary arteries in 23, and one coronary artery in 5. Ultrasound imaging detected coronary atherosclerosis (intimal thickness ≥0.5 mm) in 28 patients (56%). However, the angiography was abnormal in only 13 patients (26%). The sensitivity and specificity of coronary angiography were 43% and 95%, respectively. With ultrasound, the average atherosclerotic plaque thickness was 1.3±0.6 mm and the cross-sectional area narrowing was 34±16%. Atherosclerotic involvement frequently was focal (85%), eccentric (mean eccentricity index, 87±8), and near arterial bifurcations. Donors of the transplant recipients with coronary atherosclerosis were older than those without atherosclerosis (37±12 versus 25±10 years, P=.001). Maximal intimal thickness correlated with donor age (r=.54, P=.0001). Multivariate analysis demonstrated that donor age (P=.0001), male sex of donor (P=.0006), and recipient age (P=.03) were independent predictors of atherosclerosis. *Conclusions* Coronary atherosclerosis is frequently but inadvertently transmitted by means of cardiac transplantation from the donor to the recipient. Long-term outcomes of donor-transmitted coronary artery disease will require further evaluation.

Key Words: transplantation ■ coronary disease ■ ultrasonics

Survival rates after cardiac transplantation have improved steadily during the past 2 decades. Presently, 1-year survival rates average 80% to 90% at most active centers.[1][2] After the first year after transplantation, cardiac-allograft vasculopathy represents the principal cause of death.[3][4] Necropsy examinations have described cardiac-allograft vasculopathy as a diffuse, obliterative process characterized by concentric intimal proliferation.[5][6][7] Prior reports[8][9] have

emphasized that the predominant mechanism for genesis of transplant vasculopathy is immune injury with resulting intimal hyperplasia. Transplant coronary disease eventually results in multiple myocardial infarctions, which lead to accelerated graft failure or sudden cardiac death.

Both postmortem and intravascular ultrasound imaging studies have demonstrated that angiography underestimates the extent and severity of transplant vasculopathy.[10] [11] [12] [13] Although most centers perform routine surveillance angiography at annual catheterization, this screening process often fails to detect coronary disease. There is limited information regarding the prevalence of atherosclerosis in donor hearts and its effect on coronary disease after transplantation. Accordingly, we examined this phenomenon by performing quantitative coronary angiography and intravascular ultrasound imaging soon after cardiac transplantation. We sought to determine the frequency and morphological patterns of atherosclerosis transmitted from heart donors to recipients.

METHODS

Patient Population

The study group consisted of patients who underwent cardiac transplantation between December 31, 1992, and February 14, 1994. Patients who were ineligible for cardiac catheterization, who died during hospitalization, or who did not give informed consent were excluded. The study protocol was approved by the Institutional Review Board of The Cleveland Clinic Foundation.

Cardiac Catheterization

Recipients were studied within 2 months after cardiac transplantation. All patients underwent right and left heart catheterization, endomyocardial biopsy, coronary angiography, and intravascular ultrasound imaging. After nitroglycerin was administered sublingually, coronary arteriography was performed with large-lumen 7F coronary-guiding catheters.[14] Multiple angiographic views were obtained for optimal visualization of the coronary arteries.

Coronary Intravascular Ultrasound Imaging

Intravascular ultrasound imaging was performed on the recipients with a 30 MHz 3.5F ultrasound catheter (Boston Scientific) interfaced with a dedicated scanner (Hewlett Packard). The ultrasound catheter consisted of a 135-cm-long monorail device with a transducer enclosed in an acoustically transparent housing that was 20 mm from the tip. A driveshaft cable rotated the transducer at 1800 rpm to generate a 360° imaging plane angled 15° forward from perpendicular to the long axis of the catheter. The axial resolution of the imaging system, which varied with distance, averaged 80 to 100 µm, whereas lateral resolution ranged from 150 to 200 µm. This device generated ultrasound images at 30 frames per second, and continuously recorded these images on 1/2-in Super-VHS videotape.

After coronary angiography, recipients were given 3000 to 5000 U IV heparin before being examined by intravascular ultrasound imaging. The operator used fluoroscopic guidance to place a 0.014-in high-torque angioplasty guide wire at a distal location in the target vessel. The ultrasound catheter was placed over the guide wire at the most distal site in the coronary artery to which it could be advanced safely. The ultrasound catheter was then withdrawn gradually from this distal location during continuous imaging. At sites of atherosclerosis and adjacent normal segments, pullback was paused for identification. A cineangiogram and an audio recording documented the location of the imaging probe and its proximity to branches and other anatomic landmarks at each of these sites. Proximal, mid, and distal segments of the three major epicardial

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coronary arteries, defined according to Coronary Artery Surgery Study (CASS) classifications, were targeted for imaging.[15]

Ultrasound Imaging Analysis

The intravascular ultrasound images were analyzed by blinded observers in the intravascular ultrasound imaging core laboratory. For each site examined, a short segment (10 to 20 seconds) of videotape was digitized at 30 frames per second into a 640×480-pixel matrix image with a 24-bit gray scale. The full-motion sequence was examined frame by frame to select for analysis the image with the most atherosclerosis.

The selected frames were used to make the following measurements: (1) Maximal intimal thickness was measured as the greatest distance from the intimal leading edge to media-adventitia border, (2) minimal intimal thickness as the shortest distance from the intimal leading edge to media-adventitia border, (3) minimal luminal diameter as the shortest distance between opposing intimal leading edges, (4) lumen area as the area within the boundaries of the intimal leading edge, (5) vessel area as the area within the media-adventitia border, and (6) plaque cross-sectional area as the difference between vessel and lumen areas. Also, a relative measure of ultrasound percent area reduction was computed as follows: lumen area divided by vessel area multiplied by 100 (Fig 1).

Patients were divided into atherosclerotic and nonatherosclerotic subgroups that were stratified by the maximal intimal thickness of all examined sites. The atherosclerotic group included patients with intimal thickness ≥0.5 mm, whereas the nonatherosclerotic group included those with a maximal intimal thickness <0.5 mm. The distribution pattern of atherosclerosis was assessed longitudinally and circumferentially. Diffuse disease was defined as intimal thickening of the entire length of the artery, whereas focal disease consisted of intimal thickening of isolated sites. The circumferential distribution of atherosclerotic plaque was determined as follows.

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Angiographic Analysis

Cineangiograms were reviewed in the core cineangiography laboratory by an experienced angiographer blinded to the results of the ultrasound imaging study. The angiograms were projected onto a screen at a fixed distance with a rear-projection system (Tagarno 35AX). For each coronary artery, the CASS segment classification system was used to identify the most distal site imaged by ultrasound. Stenosis severity at sites showing any luminal narrowing was measured with an Atari digital caliper system (Sandhill Scientific, Inc). For each identifiable lesion, the operator determined vessel diameter at the stenosis and at an adjacent angiographically normal reference site to quantify percent diameter stenosis.

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Statistical Analysis

Normally distributed data were reported as mean±SD. χ^2- test or Fisher's exact test was used to find significant associations between categorical variables. An unpaired t test was used to test for differences between the mean values for continuous variables in subgroups. Pearson's correlations were used to test for relations between continuous variables. Stepwise linear regression was used to determine which factors (age, sex, cytomegalovirus [CMV] virus titers for donors and recipients, ischemic time for the donor heart, rejection episodes, donor family history for coronary artery disease, smoking history, and hypertension) were significantly related to atherosclerosis after adjustment for other significant factors. A value of $P \geq .05$ was considered statistically significant.

RESULTS

Patient Characteristics

Sixty-two adult patients underwent cardiac transplantation between December 30, 1992, and February 14, 1994. All but 5 underwent cardiac catheterization within 2 months after transplantation. The 5 patients not examined included 4 who died soon after the operation (1 from multiorgan failure, 1 from cerebral hemorrhage, 1 from sepsis, and 1 from aortic rupture). One additional patient had severe medical problems precluding catheterization. Of the remaining 57 patients, 50 underwent successful intravascular ultrasound imaging. The 7 patients who did not undergo imaging included 3 patients with scheduling constraints, 2 with angiographically evident severe coronary artery disease, and 2 who experienced technical problems related to the equipment. The 50 cardiac transplant recipients were studied an average of 4.6±2.6 weeks after the operation. This cohort included 40 men and 10 women with a mean age of 53±9 years. Of the 50 recipients, 40 had positive CMV titers and 14 had experienced at least one episode of rejection requiring treatment before catheterization (Table 1).

The donor population consisted of 30 men and 20 women with a mean age of 32±12 years. The ischemic time for the donor heart, defined as the interval between removal of the donor heart and transplantation, averaged 134±40 minutes. Of the 50 donors, 31 had positive CMV titers. The medical and social histories of donors were not complete in some cases. Twenty-four of 42 donors were known smokers, 4 of 42 were hypertensive, and 3 of 24 had a family history of coronary artery disease. Because most donors were identified after major trauma, reliable basal lipid levels were not available (Table 1).

All three major epicardial coronary arteries were imaged successfully in 22 patients, two arteries were imaged in 23 patients, and only one vessel was imaged in 5 patients (Table 2). Thus, 117 first-order epicardial vessels were examined in the 50 patients. The right coronary artery was not imaged in 19 patients, the left circumflex in 12, and the left anterior descending in 2. Arteries were not imaged in 14 patients because of tortuosity, in 8 because of a small-caliber vessel, in 5 because of a nondominant right coronary artery, in 4 because of coronary spasm, and in 2 for technical reasons. Of the 450 CASS segments targeted, 255 (112 proximal, 96 mid, and 47 distal) were imaged (Table 2), with no complications other than reversible coronary spasm.

Coronary Atherosclerosis

In 22 patients, the appearance of the coronary arteries with ultrasound imaging was normal, with maximal intimal thickness <0.5 mm (Fig 2). By applying a criterion for atherosclerosis requiring an intimal thickness ≥0.5 mm, it was determined that 28 patients (56%) had unequivocal evidence of disease (Fig 3). In this subgroup, maximal intimal thickness averaged 1.3±0.6 mm; mean luminal diameter, 3.4±1.1 mm; and average luminal cross-sectional area, 11.8±5.8 mm^2. The plaque

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cross-sectional area averaged 5.5±2.4 mm^2, which represented a cross-sectional area reduction of 34±16%. Thus, a significant amount of atherosclerosis was detected in more than half the recipients studied early after transplantation.

Distribution of Atherosclerosis

Atherosclerotic plaque involvement was focal rather than diffuse in 24 of 28 patients. In patients with focal disease, plaque was observed most frequently near arterial bifurcation sites (Fig 4). In 14 patients (50%), atherosclerotic involvement was evident near the left anterior descending–circumflex artery bifurcation. Of the 14 patients, 9 had plaque on the left anterior descending side of the bifurcation, 3 on the circumflex side, and 2 on both sides. In all cases, the plaque was on the wall opposite the arterial carina of the branching vessel. An atheroma was found near the left anterior descending–diagonal artery bifurcation in 8 patients, and plaque was seen at the circumflex-obtuse marginal bifurcation in 2 patients. One patient had a plaque in the right coronary artery at the site of bifurcation in a right ventricular branch. These patterns served to emphasize the focal nature of plaque distribution in patients studied early after transplantation. Of the 28 patients with atherosclerosis, 19 had an atherosclerotic plaque in more than one arterial segment.

A distinctly eccentric pattern of atherosclerosis was evident in these 28 patients (Fig 5). The mean eccentricity index was 87±8%, which demonstrated that nearly all of the plaque was located on one side of the artery. Of the 28 recipients who had atherosclerotic involvement, the eccentricity index was >50% in every patient and was >75% in 26 of 28. In all cases, the surfaces of the coronary plaques were smooth. There was no evidence of ulcerated plaque or plaque dissection in any of the imaged arteries. Calcified elements occurred in the plaques of 9 of 28 patients.

Risk Factors for Atherosclerosis

Donor age and recipient age were the only variables found to be significantly different between the atherosclerotic and nonatherosclerotic groups by univariate analysis. Although all donors were relatively young, the donors for recipients who had atherosclerotic involvement were significantly older (37±12 versus 25±10 years, *P*=.0003, Table 1). Linear-regression analysis revealed a moderate correlation between maximal plaque thickness and donor age (*r*=.54, *P*=.0001, Fig 6). A less-significant difference existed between the two subgroups with regard to recipient age (55±9 versus 49±10 years, *P*=.02). Univariate analysis demonstrated no significant differences for the remaining variables, including donor hypertension, smoking history, family history of coronary disease, rejection episodes, donor and recipient CMV titers, time between transplantation and imaging, ischemic time, and number of vessels and segments imaged.

Multivariate analysis demonstrated that only donor age (*P*=.0001), male donor sex (*P*=.0006), and recipient age (*P*=.03) were independent predictors of atherosclerosis. There was no correlation between recipient and donor ages (*r*=.11, *P*=.43) to explain these findings. However, when plaque thickness was analyzed with donor age and recipient age as categorical variables (age <30 or ≥30) rather than as continuous factors, only donor age (*P*=.0001) and donor sex were significant factors for predicting atherosclerosis (*P*=.008, Table 3).

Angiographic Analysis

Quantitative coronary arteriography revealed completely normal coronary arteries in 37 of 50 patients (74%). In the other 13 patients, angiographic stenosis ranged from 10% to 38% (22±7%), and ultrasound imaging confirmed the presence of a plaque in abnormal segments in 12 of these patients. Thus, coronary angiography detected atherosclerosis in only 12 of the 28 patients (43%

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sensitivity) who had atherosclerosis but correctly identified 21 of 22 normal cases (95% specificity, Fig 7). In 3 patients, angiography detected more than one lesion; some of these lesions were in segments not imaged by ultrasound. However, in all 3 of these patients, ultrasound imaging identified atherosclerosis in more than one segment.

DISCUSSION

Coronary artery disease represents the major cause of late death in patients after cardiac transplantation. Cardiac-allograft vasculopathy has been reported[16][17] to be an immunologic process in which intimal injury leads to proliferation and, eventually, to arterial obstruction. The present study, which uses quantitative coronary arteriography and a new high-resolution intravascular ultrasound imaging device, demonstrates an alternative cause. In the first few weeks after transplantation, comprehensive imaging of multiple major epicardial coronary arterial segments revealed typical atherosclerotic plaque in 56% of recipients. Thus, unequivocal evidence of transmission of atherosclerosis from the donor to recipient was present in more than one half of the recipients.

These findings are particularly striking when one considers the conservative definition that is used in the present study to classify the presence or absence of atherosclerosis. Although the normal intima in young subjects consists of only a few cell layers, limited data are available to describe the range of normal intimal thickness in adults. In a necropsy study, normal intimal thickness averaged 0.21 mm (0.10 to 0.28 mm) in 21- to 25-year-old men and 0.25 mm (0.18 to 0.35 mm) in 36- to 40-year-old men.[18] In a comparative ultrasound-histology study, patients with no known coronary artery disease had intimal thicknesses averaging 0.24±0.11 mm.[19] An in vivo ultrasound imaging study[20] reported mean intimal thickness in a "normal" population as 0.18 mm, with 95% confidence intervals of 0.06 and 0.30 mm. However, in normal subjects, intimal thickness and echogenicity increases with age. Thus, in very young subjects, a distinct laminar structure often is not evident by intravascular ultrasound.[19][20] To avoid potential controversy regarding interpretation of this study, we used a high threshold (≥0.5 mm) for abnormal intimal thickness. This value represents intimal thicknesses at least three SDs greater than any published range of normal values.

The pattern of atherosclerotic plaque in these patients was typical of atherosclerosis and distinctly different from immune-mediated transplant vasculopathy. Most atheromas were highly eccentric, with >87% of plaque on one side of the vessel. In addition, the lesions showed a predilection for sites of major bifurcations. Experimental studies suggest that low shear stress along the non–flow-dividing wall might be an important localizing force, in contrast to the high shear stress along the flow-dividing wall (carina at vessel bifurcation).[21] The morphological characteristics of the plaques are typical of conventional atherosclerosis and dissimilar to vasculopathy as described in the transplant population.[22] Indeed, features characteristic of transplant vasculopathy, particularly diffuse, concentric intimal thickening, were absent in our study group. Thus, it is evident that the atherosclerotic changes were present in the donor hearts and transmitted to the recipients at cardiac transplantation.

This study highlights the limitations of standard coronary arteriography for detecting early transplant coronary disease. Quantitative coronary angiography was relatively insensitive, revealing less than half the lesions identified by intravascular ultrasound imaging. An important explanation for the discrepancy between angiography and ultrasound imaging is provided by the anatomic pattern of early atherosclerosis. As originally described by Glagov et al,[23] early coronary disease is characterized by remodeling of the coronary artery, which protects against luminal encroachment of the atherosclerotic plaque. Thus, segments with major intimal thickening retain a

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lumen size virtually identical to adjacent, uninvolved sites. In the absence of luminal narrowing, angiography fails to detect early disease.

Few demographic characteristics were useful for predicting the likelihood of donor atherosclerosis. Multiple-regression analysis showed only donor age, recipient age, and donor sex to be independent predictors. The strongest predictor was donor age, which correlated significantly with intimal thickness. Although donor sex was not (univariately) associated with maximal intimal thickness, it became a significant factor after adjusting for donor age. It is not surprising that hearts from older men tended to have a higher prevalence of atherosclerosis, since age and male sex are well-known risk factors for coronary disease. It is more difficult to explain the unfavorable effect of recipient age on atherosclerosis early after transplantation. Acute rejection,[24] [25] CMV infection,[26] [27] and conventional risk factors have been suggested to be possible contributors to the development of chronic transplant coronary disease, but were not significant in this study. However, information about donor hypertension, smoking, family history, and hypercholesterolemia was incomplete. Thus, a full understanding of the relation between donor risk factors and early atherosclerosis cannot be stated conclusively.

In a recent report from St Goar et al,[28] with a larger (4.3F) earlier-generation intravascular ultrasound catheter, 25 patients were studied within 1 month of cardiac transplantation. Imaging was limited to segments extending from the ostium of the left main coronary artery to the midportion of the left anterior descending coronary artery. In 5 of 25 patients, there was eccentric intimal thickening >0.5 mm. The difference in the prevalence of atherosclerosis between the study by St Goar et al and ours is explained by several factors. In the present study, imaging was more extensive and an improved, low-profile 3.5F ultrasound probe was used. This permitted examination of multiple segments from major epicardial coronary arteries in 86% of patients. At least two of the three major coronary arteries were imaged in 90% of recipients. In addition, our study population was larger and donors were older (32±12 versus 28±8 years old).

These in vivo findings provide strong evidence as to the high prevalence of coronary atherosclerosis in young and middle-aged Americans. These findings are consistent with autopsy studies performed on large populations of trauma victims during the Korean and Vietnam wars.[29] [30] During the Korean War, atherosclerotic plaques with a wide range of stenoses (10% to 90%) were found in 117 of 300 young soldiers.[29] Autopsy studies on American soldiers killed in Vietnam revealed that 45% had some coronary atherosclerosis.[30] In both necropsy studies, all the deceased were men and mean subject age was 22 years old, whereas in our study, 40% of the donors were women and the mean donor age was 32±12 years. Furthermore, necropsy studies, which often examine explanted vessels not distended by physiological pressures, represent only estimations of the severity of coronary disease.

This intravascular ultrasound imaging study provides direct in vivo evidence of occult, but rather extensive, atherosclerosis in a young and presumably healthy population. Without high-resolution intravascular ultrasound imaging, detection and quantification of atherosclerosis would not have been possible in living humans. Ultrasound imaging provides unique cross-sectional information that is analogous to pathology and that cannot be obtained in coronary arteries from any other imaging technique.

The older age of the donor population in this study reflects an important trend in cardiac transplantation. The limited availability of suitable donors and the long waiting lists for transplantation have increased the use of older donors.[31] However, the presence of atherosclerosis in more than 80% of our donors who are ≥30 years old indicates a potential hazard to this approach. The presence of coronary atherosclerosis in so many transplant recipients raises an important clinical question. Does disease transmitted from the donor increase

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the risk of accelerated transplant vasculopathy? This important clinical question cannot be answered until angiographic, ultrasound, and clinical follow-up studies determine the long-term prognostic significance of transmitted coronary disease.
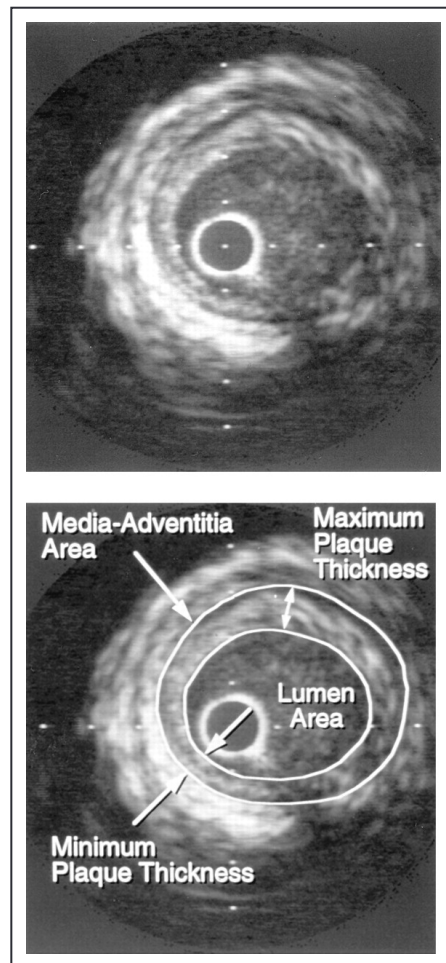
Figure 1.   Measurements of lumen and vessel wall dimensions in an ultrasound image.
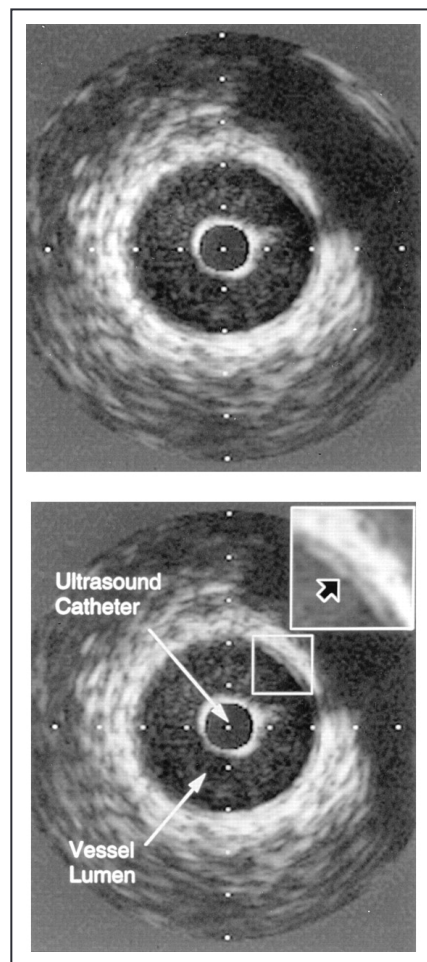
Figure 2. Ultrasound image of a normal coronary artery. Inset shows the three layers of the vessel wall (black arrow). The thin echogenic inner layer corresponds to the intima, the thin echolucent middle layer to the media, and the echogenic outer layer to the adventitia.
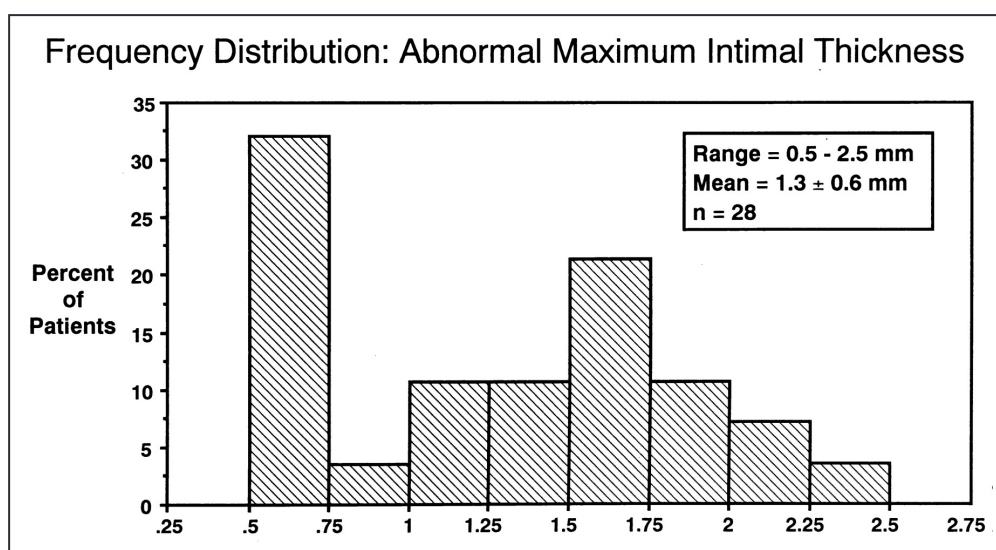
Figure 3. Bar graph showing distribution of intimal thickness in 50 cardiac transplantation patients.

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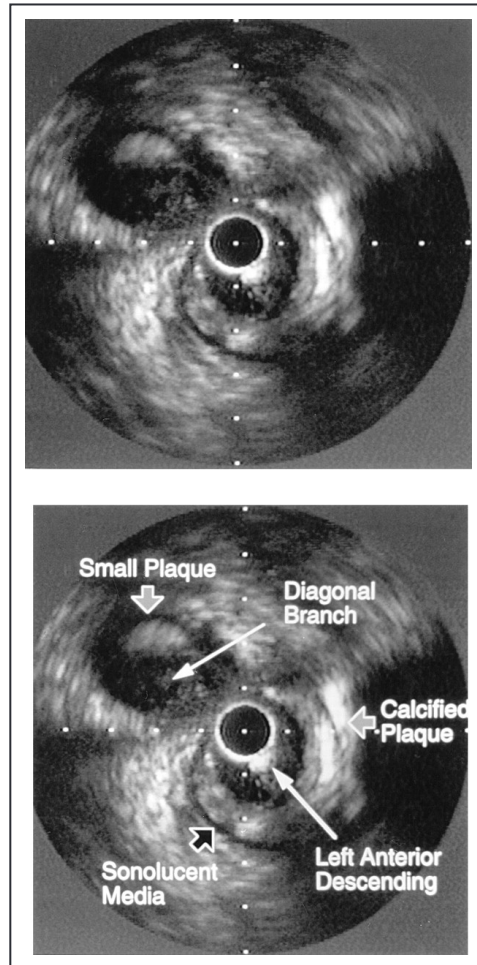
Figure 4.   Ultrasound image of an eccentric, partially calcified atherosclerotic plaque in the left anterior descending artery at the bifurcation site of the major diagonal branch. A small eccentric plaque is seen in the diagonal branch opposite the flow-dividing wall.

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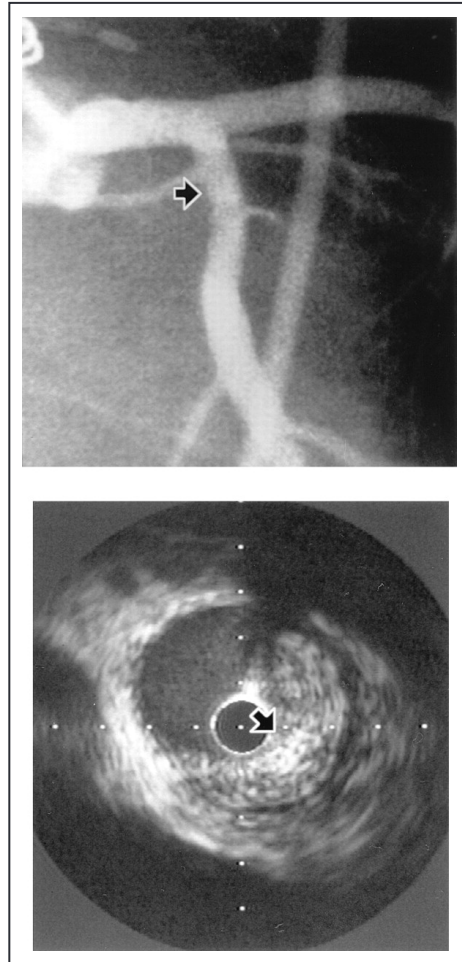
Figure 5.   Top, Angiogram of the left coronary artery in the right anterior oblique projection showing mild luminal narrowing. Black arrow indicates the site of intravascular ultrasound imaging. Bottom, Two-dimensional intravascular ultrasound image revealing a large eccentric plaque in the proximal circumflex artery (black arrow).
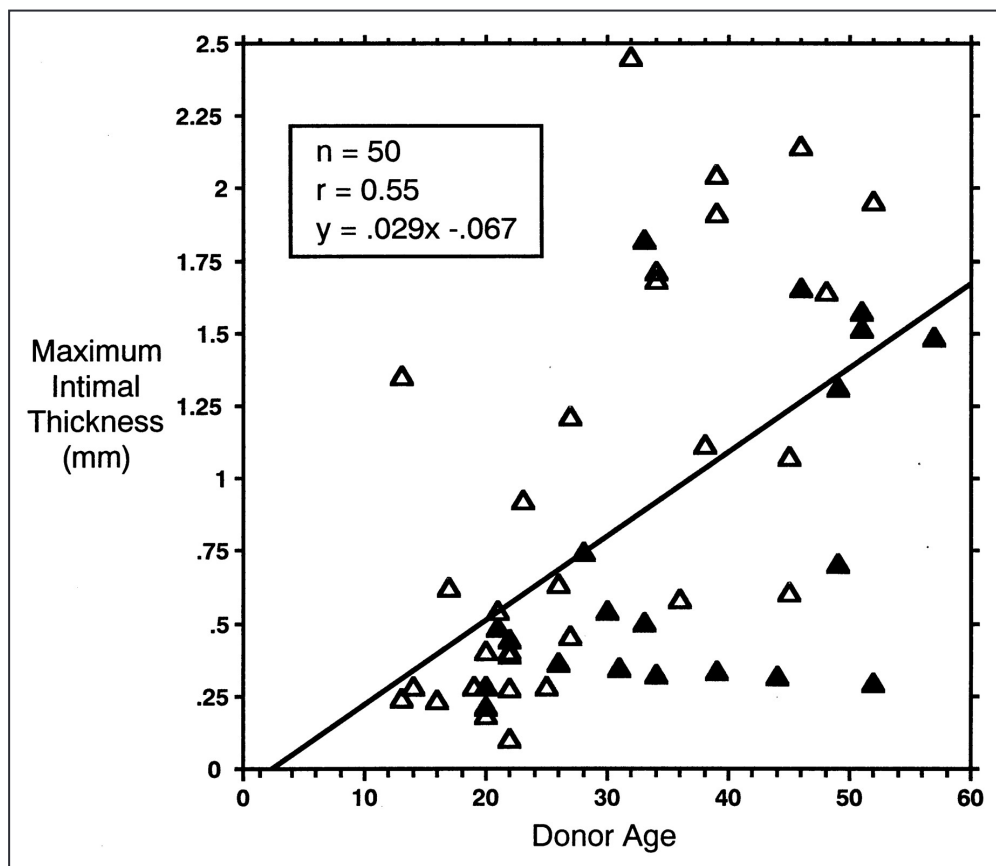
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Figure 6. Scatterplot showing correlation between donor age and intimal thickness. ᵒ indicates male donors; ▪, female donors.
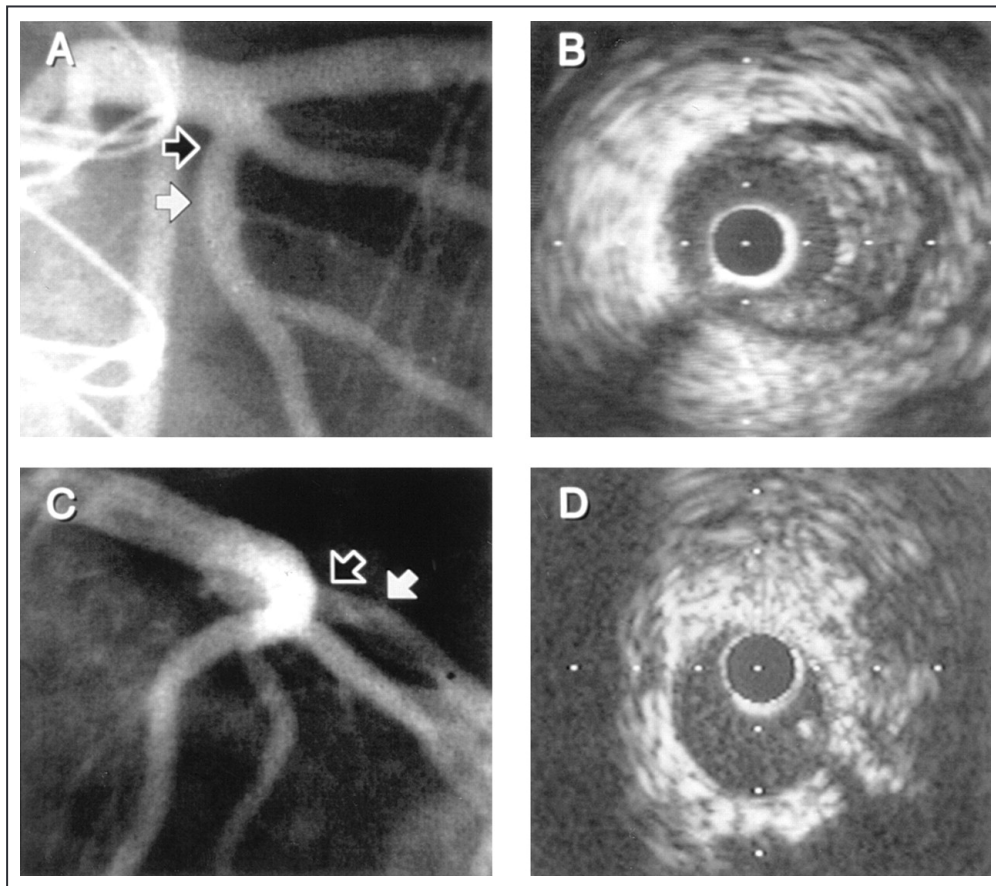
Figure 7. A, Arteriogram of the left coronary artery in the right anterior oblique projection. Black arrow indicates imaging site shown in B; white arrow, imaging site shown in D. B, intravascular ultrasound image of the proximal circumflex artery. Despite a large eccentric plaque, a large circular lumen similar to that in D is preserved because of remodeling. C, Left coronary angiogram in the left anterior oblique projection. Black arrow indicates imaging site shown in B; white arrow, imaging site of proximal circumflex artery shown in D. D, intravascular ultrasound image at another site in the proximal circumflex artery showing normal arterial morphology.

Table 1. Demographic and Clinical Characteristics of the Donor and Recipient Populations (Table view)

	All Patients	Intimal Thickness		Univariate P	Multivariate P
		<0.5 mm	≥0.5 mm		
Donor					
Age, y (mean±SD)	32±12	25±10	37±12	.0002	.0001
Male sex, n (%)	30 (60)	12 (55)	18 (64)	.49	.0006
Smoking, n (%)[1]	24/42 (48)	10/18 (45)	13/24 (46)	.99	
Hypertension, n (%)[1]	4/42 (10)	1/20 (5)	3/22 (14)	.62	
Family history of CAD, n (%)[1]	3/24 (13)	1/10 (10)	2/14 (14)	.81	
CMV titers	31 (63)	15 (68)	16 (59)	.52	
Recipient					
Age, y	52±10	49±10	55±8	.02	.03
Male sex, n (%)	40 (80)	16 (73)	24 (86)	.25	
CMV titers, n (%)	40 (80)	17 (77)	23 (82)	.73	
Rejection, n (%)	14 (28)	8 (36)	6 (21)	.24	
Ischemic time, minutes (mean±SD)	134±40	125.5±39	140.6±39.8	.18	
Imaging time, weeks (mean±SD)	4.6±2.5	4.4±1.8	4.7±3.0	.61	

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CAD indicates coronary artery disease; CMV, cytomegalovirus.
[1] The values in the denominator indicate no. of patients for whom information was available.

Table 2. Number of Major Epicardial Vessels and Arterial Segments Imaged by Intravascular Ultrasound (Table view)

	All Patients	Intimal Thickness		P
		<0.5 mm	≥0.5 mm	
No. of vessels imaged, no. of patients (%)				
1	5 (10)	3 (14)	3 (11)	
2	23 (46)	9 (41)	14 (50)	
3	22 (44)	10 (45)	12 (43)	.38
No. of segments imaged, n (%)[1]				
Proximal	112/150 (75)	51/66 (77)	61/84 (73)	
Mid	96/150 (64)	43/66 (65)	53/84 (63)	
Distal	47/150 (31)	21/66 (32)	26/84 (31)	.49
Total	255/450 (57)	115/198 (58)	140/252 (56)	

[1] The values in the numerator are the number of segments that were imaged. The values in the denominator are the number of segments that were potential targets for imaging.

Table 3. Coronary Atherosclerosis in Various Donor Age Groups (Table view)

Donor Age	No. of Patients With Atherosclerosis/Total Patients (%)	
<20	2/6 (33)	
20-29	5/18 (28)	
30-39	10/13 (77)	
40-49	7/8 (88)	
≥50	4/5 (80)	

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Acknowledgments

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A0306

Determination of Cutoff Score for a Diagnostic Test

G Singh

Citation

G Singh. *Determination of Cutoff Score for a Diagnostic Test*. The Internet Journal of Laboratory Medicine. 2006 Volume 2 Number 1.

Abstract

The cutoff value for a new diagnostic test for classifying cases as positive or negative may be determined utilizing some statistical techniques also in addition to clinical and other criteria. Mean ± 2SD, ROC curve, discriminant score methods may prove to be useful statistical tools for such situation.

When a new diagnostic test is developed or when a diagnostic test is to be used in a clinical condition different from the one for which the test was developed, test's cutoff score may require re-determination. This determination or re-determination may usually be based on biological, clinical or demographic situations. Some statistical methods may also be used or may be used in addition to the clinical experiences, analytical and empirical evidences for finding more reliable and valid cutoff point for classifying cases as positive or negative. 95% confidence interval (CI) of mean i.e. Mean ± 2SD method, ROC curve, discriminant function analysis may prove to be helpful statistical tools for such situation.

MEAN ± 2SD

An easy, crude and commonly used method is the application of 95%CI of mean for choosing a cutoff. A sample of adequate size of diagnosed cases (known positive cases) suffering from particular disease may be chosen. Then the diagnostic test is administered and values observed for cases are recorded. Mean and standard deviation (SD) of test values are calculated. Now an interval obtained by subtracting 2 x SD from mean and by adding 2 x SD to mean (that is, $\mu \pm 2\sigma$) shows that the chance of a test value coming outside this interval will be less than 5%. The lower limit of this interval (i.e. mean - 2SD) may be considered as cutoff point. If a subject's test value comes less than this cutoff then may be considered negative (normal) and if value comes greater than or equal to cutoff value then considered positive (diseased). This method may carry a chance of declaring some false negative cases, which can lower its sensitivity. Alternatively, this method may be carried out on a sample of known negative cases. In this case the upper limit of its

95%CI (i.e. mean + 2SD) may be taken as cutoff value. If a subject's test value comes greater than this cutoff value then may be considered positive (diseased). This approach may carry a chance of declaring some false positive cases that can lower its specificity. Depending upon the seriousness of the loss that may incur on lower sensitivity or specificity, a suitable approach may be chosen.

For illustration, take an example of CA-125 a glycoprotein, which is commonly used tumor marker in ovarian carcinoma. In a small study[1] mean ± sd of CA-125 for healthy volunteers was reported 8.08 ± 3.26 U/ml. Assuming if it is a large sample and drawn randomly from a specific population then, 95%CI comes 1.56 to 14.60 (i.e. 8.08 – 2x3.26 to 8.08 + 2x3.26). This shows that the chance of the value of CA-125 in such situation coming outside the interval 1.56 to 14.60 is less than 5%. In simple words, there is a high chance that test value for such healthy subjects may come in between 1.56 to 14.60. Now the upper limit of this interval i.e. 14.60 may be taken as a cutoff score. To determine cutoff score of this marker for specific diseased group, mean and sd of randomly drawn large sample from such diseased population can be used to obtain 95% confidence interval whose lower limit may be chosen as cutoff value. A subject having higher value than this cutoff may be considered as positive (diseased).

This method may work as a preliminary exercise in determining cutoff score of a test under varying conditions. The drawback here is that upper limit found for negative (healthy) subjects may not coincide with the lower limit found for positive (diseased) cases. Sometimes there may be a gap between the two and sometimes they overlap.

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Determination of Cutoff Score for a Diagnostic Test

ROC CURVE

Receiver operating characteristics (ROC) curve between sensitivity and 1-specificity is a useful method to evaluate the performance of a diagnostic test in classification of subjects into two categories (say) positive and negative.[2] ROC curve may be used to judge how well the test performs. If area under the curve is near 1 it has higher chance of correct classification and when it is near 0, higher chance of incorrectly classifying in opposite group. The value 0.5 shows the test is no better than just tossing a coin for classification into positive or negative.[3] Some statistical software (like SPSS ver.10 or onwards) may be used for ROC curve. For determination of cutoff value this method may also be used. A sample of adequate size may be taken with known positive and negative cases. Then administer the diagnostic test and note the values observed. For every observed value this method displays sensitivity and 1-specificity of the test. Now one may choose a particular observed value of the test as cutoff value, which corresponds to the desired sensitivity and specificity (or 1-specificity).

In a study[4] to determine the cutoff value of serum I-CaD determined by ELISA for differentiating between patients with or without a glioma, a receiver-operating characteristics analysis was done. Taking each observed value as cutoff, ROC gives sensitivity and specificity of the test. This study reported the following result;

Figure 1

Serum *I*-CaD	Sensitivity	Specificity
Test values observed		
-	-	-
-	-	-
40	91 %	82 %
-	-	-
45	91 %	84 %
-	-	-
50	88 %	85 %
-	-	-
-	-	-

It is to be seen here that when 50 is taken as cutoff sensitivity decreased though specificity increased slightly. So a serum I-CaD value 45 may be thought of optimally better cutoff for differentiating patients. However, in a situation where high sensitivity is more desirable than high specificity, one may look for cutoff value even smaller than 40 similarly, when high specificity is more important, cutoff value greater than 50 may be chosen for classification.

A high sensitivity results in low number of false negative cases while high specificity leads to low number of false positive cases. Therefore, depending on the situation, requirement and seriousness of loss due to misclassification optimal value of sensitivity and specificity is decided and the test value corresponding to this may be taken as cutoff score for classification.

DISCRIMINANT SCORE

Discriminant function analysis is a popular tool in solving classification problem. A function is generated from a sample of known positive and negative cases then, the function is used for new cases with observed diagnostic test values to classify them as positive or negative. This method gives for each case discriminant score and predicted group membership corresponding to the observed test value. More elaborately, let a sample of known negative (normal) and positive (suffering with the particular disease) cases be chosen. The diagnostic test (for which cutoff is to be determined) may now be administered and the test value observed may be recorded. Perform discriminant function analysis using some statistical software. Observed test values will be entered in one column and known group membership (positive or negative) in another column. Result of the analysis will display discriminant score and predicted group membership for each case. If the test is such that low value indicates normal (negative) and high value as disease (positive) then, sort the cases according to discriminant score in ascending order. When it is arranged in increasing order of discriminant score, locate the case (row) from where the predicted group membership changes. The test value corresponding to this discriminant score from where predicted group membership changes (from normal to disease) might be taken as cutoff score. A value coming greater than or equal to this cutoff will be treated as positive while less than cutoff value as negative. If a test is such that low value indicates disease (positive) and high value as normal (negative) then also this method can be used to find a cutoff. Discriminant score may prove to be a suitable method in such type of situation requiring determination of cutoff value of a diagnostic test. It is also to be mentioned here that this method may be used for determining cutoff scores of more than one diagnostic test administered at a time. That is, when classification is to be made based on scores of more than one diagnostic tests together.

Lastly, it is worth mentioning here that determination of cutoff value may not be considered as prerogative of a statistician. A value derived by analyzing data may

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sometime be clinically unacceptable on certain grounds.

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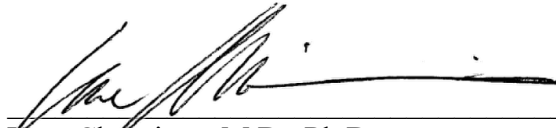
IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CAREDX, INC.,	)	
Plaintiff,	)	
	)	
v.	)	
	)	C.A. No. 1:19-662 (CFC) (CJB)
NATERA, INC.,	)	
Defendant.	)	
	)	
	)	
	)	

REBUTTAL EXPERT REPORT OF UWE CHRISTIANS, M.D., Ph.D.

October 16, 2020

CONTAINS INFORMATION MARKED OUTSIDE ATTORNEYS' EYES ONLY

_____   October 16, 2020
Uwe Christians, M.D., Ph.D.   Date
Department of Anesthesiology
University of Colorado

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for this statement. *Id*. at 4. In fact, the data at the 1.0% cut-off used by both CareDx and Natera suggests otherwise as Prospera correctly identified 6 of 11 cases of rejection compared to only 5 of 11 by AlloSure. *Id*. at 8, Table 2 (using sensitivity and 11 samples to calculate results).

VI.   Opinions

89.   For the reasons set forth below, it is my opinion that Natera's Sigdel study was conducted in a reasonably reliable manner and provides a scientifically sound basis for the clinical performance metrics reported in the Sigdel Paper. Further, it is my opinion that, while not identical, the study designs, protocols, and demographics for the studies reported in Sigdel and Bloom is sufficiently similar to allow for a reasonable and scientifically sound comparison between the clinical performance characteristics (*e.g.*, sensitivity, AUC, and NPV) of Prospera in Sigdel with that of AlloSure in Bloom. My opinions are further bolstered by Sigdel and Bloom being cited and compared by other scientists in the field as well as MolDx's approval for Medicare reimbursement of Prospera, despite CareDx's multiple presentations (directly and indirectly) of variations of the arguments and opinions that appear in Dr. Weisbord's Opening Report, as well as Dr. Weisbord's failure to cite to any criticisms of the Sigdel paper from independent members in the transplantation and nephrology fields. Also, I opine that the Natera advertising claims that Dr. Weisbord explicitly identifies in his Opening Report are not false or misleading for a variety of reasons. Finally, I comment on Dr. Weisbord's propensity for misinterpretation and selective editing of Natera documents and witness testimony that form the main, if not sole, bases for his opinions.

A.   The Sigdel Study was Reliably Conducted and Provides a Reasonable Basis for the Clinical Validation of Prospera®.

90.   Dr. Weisbord levies a number of alleged criticisms of the study in the Sigdel Paper. *See, e.g.,* Weisbord Op. Rep. at ¶¶ 51-76. However, I disagree with Dr. Weisbord's opinions and

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(c) allow for a reasonable comparison of the performance parameters reported in Sigdel for Prospera to those reported in Bloom for AlloSure, as discussed more fully in the next section.

B.   Natera's Comparisons of Prospera and AlloSure Based on the Sigdel and Bloom Studies are Neither False, Nor Misrepresentations of the Published Data

133.   Dr. Weisbord also opines that the Bloom and Sigdel Papers cannot be directly compared. *See* Weisbord Op. Rept. at ¶¶ 77-89. As discussed below, I disagree with Dr. Weisbord for a number of reasons.

    i.   The Differences in Study Design between Sigdel and Bloom Do Not Preclude Comparison of the Performance Metrics in Those Papers

134.   Dr. Weisbord claims that there are "substantial" or "fundamental differences in study design between Sigdel and Bloom that precludes an "apples-to-apples" or "any direct" comparison of the results from these two studies. *See* Op. Rept. at ¶¶ 78-79. I disagree for at least the following reasons.

135.   First, study methodologies and designs do not have to be exactly identical, or as Dr. Weisbord colloquially refers to as "apples-to-apples"[31] (*see* Op. Rept. ¶ 78-79), to be scientifically valid. While a parallel comparison of competing assays measuring all of the samples in the same study is generally considered ideal, comparison of the diagnostic performance (*e.g.*, sensitivity, specificity, PPV, NPV, AUC) of two tests assessed in different studies is informative, scientifically valid, and commonly done. *See, e.g.*, Clark, et al., "User Protocol for Evaluation of Qualitative Test Performance: Proposed Guideline," Nat'l Comm. Clinical Lab. Standards, Vol. 20 (2000) ("Clark"). The Sigdel and Bloom studies have several key characteristics in common that make a comparison possible - both studies compare to the same gold standard (Banff classification of

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[31] This term is subjective and vague and not commonly used by clinicians. It is unclear exactly what Dr. Weisbord considers to be an "apples-to-apples" comparison based on his report. Thus, I reserve the right to amend my opinions if he clarifies what he means by this term.

biopsy histology), use the same matrix (plasma), use the same result parameter (% dd-cfDNA),

use the same cut-off (1%) and both studies are based on the target population, kidney transplant

patients

136.   Second, simply put, I do not agree with Dr. Weisbord's opinion that there are

"substantial differences in study design." Weisbord Op. Rept. at ¶ 78. The differences that do

exist between the study designs reported in Sigdel and Bloom do not make it improper to compare

performance parameters of different assays in different studies.[32] For example, it does not matter

that Sigdel was retrospective and Bloom was prospective as Dr. Weisbord opines. *See* Weisbord

Op. Rept. at ¶ 77-79. As described above, such labels are confusing and misused in the literature,

as even Dr. Weisbord's own reference (Euser) admits. Also, Dr. Weisbord is incorrect that Bloom

was fully prospective and that Sigdel was fully retrospective for the reasons previously stated in

Paragraphs 99 above. Further, different study designs are compared often and numerous third-

parties have compared the data in Bloom and Sigdel. *See, e.g.,* Zhang at 6; Andrikovics at 8;

Viklicky at 29; Salez at 256-58; Del Giorno at 169-78. In addition, Sigdel compared their results

to Bloom in their manuscript: "In a recent study that amplified hundreds of target SNPs in dd-

cfDNA to detect active rejection in kidney allografts, that method was able to discriminate AR

from non-rejection with an AUC of 0.74, 59% sensitivity, and 85% specificity [14]…." Sigdel at

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[32] In Paragraph 79, Dr. Weisbord once again misinterprets selected testimony from Dr. Billings. To the extent that we experts are allowed to opine on interpretations of Dr. Billings' testimony, I do not believe he was saying that comparing Bloom and Sigdel was a difficult thing to do. Rather, I believe he was stating that doing an exact comparison with different assays is difficult to do. *See* Billing Dep. Tr. at 216:6-22; *see also id.* at 276:8-277:7; NTRA000000025582. Dr. Billings also never testified that Bloom is the superior study design type because he only says that randomized clinical trials of all comers are the gold standard, but Bloom is not randomized and did not take all comers.

potential sources of bias, Dr. Weisbord has not proven that any such biases actually occurred in Sigdel. I do not believe any significant biases exist in Sigdel for at least the reasons in Section VI.A above.

143.   As he failed to do with racial/ethnic diversity demographic differences between Sigdel and Bloom (*see* Paragraphs 95, 137 above), Dr. Weisbord also does not explain how or provide evidence that differences in living vs. deceased donors or average age or average weight in Bloom and Sigdel preclude a reasonable comparison between the performance metrics of the two studies. I disagree with Dr. Weisbord's unsupported opinions that sensitivity, specificity, and AUC are dependent on the physical characteristics of the population in evaluating and comparing laboratory diagnostic tests. For instance, a literature search revealed that the relationship between demographics, concomitant diseases, and drug treatment and the results of dd-cfDNA tests in kidney transplant patients has not yet been systematically studied. Dr. Weisbord argues that there were "meaningful differences". However, meaningful is an ambiguous term without further explanation, which Dr. Weisbord fails to provide.   For example, Dr. Weisbord mentions the difference in weight. The mean weight in the Sigdel study was approximately 76 kg that of the Bloom study 85 kg, both with a standard deviation of around 17-21 kg, which indicates that there was a substantial overlap between the two study populations.

144.   Further, Dr. Weisbord claims that potential variations in timing between the transplant and the biopsy being analyzed and circumstances (e.g., levels of immunosuppresants and different levels of kidney function). *See* Op. Rept. at ¶ 85. I disagree. Dr. Weisbord fails to consider that both AlloSure and Prospera depend on the donor and recipient cfDNA ratios and the assay results represent a snapshot of the transplant kidney's cell integrity, or the lack thereof in case of injury at the time of blood draw. Both assays assume that the recipient background cfDNA

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levels stay rather stable and that the variable that changes and, thus, is an indicator of kidney transplant health is the dd-cfDNA. The level of recipient cfDNA is unlikely to be tightly correlated with time after transplantation but is rather influenced by temporary fluctuations that are independent of the time of transplantation such as disease, medications, lifestyle events, and environmental exposures. Dr. Weisbord speculates that levels of immunosuppressants are a potential reason for differences between the Boom and Sigdel study groups, again without providing evidence as neither manuscript reports immunosuppressant blood concentrations. Immunosuppressant exposure is tightly controlled in transplant patients. Most immunosuppressive protocols after kidney transplant patients are based on tacrolimus. Tacrolimus is dosed guided by regular therapeutic drug monitoring to result in blood concentrations in a narrow therapeutic target window, which is the same for most kidney transplant patients, and makes it highly unlikely that there were substantial differences in tacrolimus exposure in the Sigdel and Bloom study cohorts. *See, e.g.*, Brunet, *et al.*, "Therapeutic Drug Monitoring of Tacrolimus-Personalized Therapy: Second Consensus Report," Ther. Drug Monit., Vol. 41, 261-307 (2019). Further, Oellerich reported "[i]n a patient subset (N = 24) there was a significantly higher rate of patients with elevated dd-cfDNA (cp/mL) with lower tacrolimus levels (<8 μg/L) compared to the group with higher tacrolimus concentrations (P = .0036) suggesting that dd-cfDNA may detect inadequate immunosuppression resulting in subclinical graft damage." Oellerich 2019 at 3093. Under-immunosuppression and the resulting active rejection is exactly what dd-cfDNA assays are supposed to detect so that Dr. Weisbord's speculation that potential differences in immunosuppressant levels in both studies are problematic are without scientific merit.

145.   Dr. Weisbord also points to differences in the eGFR between the AR and no rejection biopsies in Sigdel and Bloom and claims this "underscores the problems comparing

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A0316

at ¶¶ 98, 99, 124-128;[43] 130-135; 137-139, 142, 144, 145, 147, 149, 151, 153, 155-157, 160-162, 165-167, 169-171, 175-177, 180-182, 185-186, 192-194, 197-199, 204-206, 210-212, 216-218, 221-224.

174.   Dr. Weisbord often characterizes Natera's statements of this nature as containing "head-to-head comparisons" of Sigdel and Bloom. *See, e.g.,* Op. Rept. at ¶¶ 98, 99, 125, 132, 137, 142, 147, 165, 169, 175, 180, 185, 192, 197, 204, 210, 216. This is not how clinicians typically use this term. Rather, any reasonable clinician reading the Natera claims would understand that Sigdel and Bloom were separate studies and did not involve head-to-head comparisons of Prospera and AlloSure. A reasonable clinician would also understand that comparing any two studies done on different cohorts, from different locations, with different assays, and other differences in methodology or protocols are not properly characterized as "head-to-head", but are still a reasonable way to compare to assays. Any reasonable physician would also understand that Natera's comparison of the performance parameters in Sigdel and Bloom is different from a "head-to-head" study design, as even Dr. Weisbord seems to recognize by describing Melancon as providing the "only head-to-head study." *See, e.g., Id.* at 198.[44]

175.   Regardless of his nomenclature, I disagree with Dr. Weisbord's opinions that claims accurately reflecting—explicitly or implicitly—the performance metrics (such as

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[43] Dr. Weisbord appears to include claims to "unparalleled precision" (¶ 125) and "most precise" (¶¶ 131-132) in this category. But, as detailed in Section VI.D.vii below, these claims are actually referring to the analytical validity studies by Altug on Prospera and Grskovic on AlloSure, not Sigdel and Bloom. Based on the lack of citation to them in his report or materials considered list, Dr. Weisbord apparently did not review or consider the Altug and Grskovic papers, which could explain his misinterpretation of Natera's "precision" claims.

[44] As discussed in Section VI.D.i.a, Dr. Weisbord's reliance on Melancon is misplaced because it is a very weak study. Further, as noted in Paragraph 164, there is other head-to-head data from UCLA that Dr. Weisbord did not consider in his opening report, which shows that Prospera performed better than AlloSure in another small study.

A0318-A0999
Intentionally Omitted

AMENDMENT UNDER 37 C.F.R. §1.116	Attorney Docket No.	STAN-706
	Confirmation No.	6003
	First Named Inventor	Quake, Stephen R.
	Application Number	13/508,318
Address to: Mail Stop AF Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Filing Date	July 19, 2012
	Group Art Unit	1639
	Examiner Name	Bunker, Amy M.
	Title: "Non-Invasive Diagnosis of Graft Rejection in Organ Transplant Patients"	

Sir:

This amendment is responsive to the Final Office Action dated November 14, 2013 for which a three-month period for response was given making this response due on or before February 14, 2014. In view of the amendments to the claims and the remarks put forth below, reconsideration and allowance are respectfully requested.

AMENDMENTS TO THE CLAIMS:

1-35. (Cancelled)

36. (Currently Amended) A method comprising:

(a) providing a sample from a subject who has received a transplant from a donor;

(b) conducting a multiplexed reaction on the sample to detect one or more circulating, cell-free nucleic acids, wherein (i) the one or more circulating, cell-free nucleic acids originated from the transplant from the donor and (ii) detecting the one or more circulating, cell-free nucleic acids comprises sequencing the one or more circulating, cell-free nucleic acids; and

(c) diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the one or more nucleic acids based on the detection of the one or more nucleic acids by the sequencing reaction, wherein the sensitivity of the method is greater than 56%.

37-38. (Cancelled)

39. (Previously Presented) The method of claim 36, wherein the one or more nucleic acids derived from the donor are detected based on a marker profile comprising one or more genetic variations selected from single nucleotide polymorphisms (SNPs), one or more variable number of tandem repeats (VNTRs), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, or one or more insertion elements.

40. (Cancelled)

41. (Previously Presented) The method of claim 36, wherein the one or more nucleic acids is DNA.

42-43. (Cancelled)

44. (Previously Presented) The method of claim 36, wherein sequencing the one or more circulating, cell-free nucleic acids comprises shotgun sequencing.

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45. (Previously Presented) The method of claim 36, wherein the multiplexed reaction occurs in a single container.

46. (Previously Presented) The method of claim 36, wherein the reaction to detect one or more nucleic acids comprises detecting at least ten different nucleic acids.

47. (Previously Presented) The method of claim 36 further comprising administering an immunosuppressive drug.

48. (Previously Presented) The method of claim 36, wherein diagnosing, predicting, or monitoring transplant status or outcome comprises treating a transplant rejection in a subject in need thereof.

49. (Previously Presented) The method of claim 36, wherein diagnosing, predicting, or monitoring transplant status or outcome comprises determining, modifying, or maintaining an immunosuppressive regimen.

50. (Previously Presented) The method of claim 36, wherein detecting the one or more circulating, cell-free nucleic acids comprises detecting genetic variations.

51. (Previously Presented) The method of claim 36, wherein detecting the one or more circulating, cell-free nucleic acids further comprises conducting an assay selected from: digital PCR, real-time PCR, array, or any combination thereof.

52. (Cancelled)

53. (Previously Presented) The method of claim 50, wherein the genetic variations are selected from single nucleotide polymorphisms (SNPs), one or more variable number of tandem repeats (VNTRs), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, one or more insertion elements, or any combination thereof.

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54. (Previously Presented) The method of claim 36, wherein the one or more nucleic acids comprise at least one single nucleotide polymorphism.

55. (Cancelled)

56. (Previously Presented) The method of claim 36, wherein the sample is blood or serum.

57. (Previously Presented) The method of claim 36, wherein the sample is urine or stool.

58-65. (Cancelled)

66. (Previously Presented) The method of 36, wherein the transplant is selected from the group consisting of: kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

67. (Previously Presented) The method of claim 36, wherein the transplant is a heart transplant or kidney transplant.

68-69. (Cancelled)

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REMARKS

Claims 36, 39, 41, 44-51, 53, 54, 56, 57, 66 and 67 are currently pending. Claims 1-35, 37, 38, 40, 42, 43, 52, 55, 58-65, 68 and 69 are cancelled. Claim 36 is currently amended. Support for the amended claim can be found in claim 42 (cancelled). No new matter has been added.

Interview Summary

Applicants greatly appreciate the courtesy that was extended by Examiner Bunker and Supervisory Patent Examiner Leavitt during the telephonic interview conducted on January 13, 2014 with Pamela Sherwood, Vern Norviel, Lucia Muntean and Stephen Quake. During the interview, differences between certain references (Moreira et al., Lo Yuk-Ming et al., and Baxter-Lowe et al.) and the claimed invention were discussed. The Office and Applicants discussed proposed amendments to clarify how the sequencing reaction is used in diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant.

35 U.S.C. § 103

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66, and 67 were rejected under 35 U.S.C. § 103(a) as being obvious over Moreira et al. in view of Lo Yuk-Ming et al. and further in view of Baxter-Lowe et al.

The pending claims recite using a sequencing reaction to detect one or more nucleic acids originating from a transplant from a donor. (See Claim 36 (b)). "The key to supporting any rejection under 35 U.S.C. 103 is the clear articulation of the reason(s) why the claimed invention would have been obvious" (*see* MPEP 2142). As the Office has not articulated why it would be obvious to one skilled in the art to use a sequencing reaction to detect donor-derived nucleic acids based on the disclosures of Moreira et al., Lo-Yuk-Ming et al., and Baxter-Lowe et al., the Office has not made a prima facie case for obviousness for claim 36. In particular, the Office relies on Moreira et al. for allegedly disclosing a shotgun sequencing instrument. However, Applicants submit that the Prism® 7000 Sequence Detection System is not a shotgun sequencer, but rather a real-time PCR instrument according to the manufacturer's web-site. In addition, Moreira et al., Lo Yuk-Ming et al., and Baxter-Lowe et al., alone or in combination, do not teach, suggest or motivate one of skill in the art to use a sequencing reaction to detect donor-derived nucleic acids.

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As discussed during the interview, claim 36(c) has been further amended to recite "diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the one or more nucleic acids based on the detection of the one or more nucleic acids by the sequencing reaction." This amendment incorporates the limitation of claim 42 into claim 36(c) and is made without conceding the validity of the rejection and solely to expedite prosecution. Moreira et al., Lo Yuk-Ming et al., and Baxter-Lowe et al., alone or in combination, do not teach, suggest or motivate one of skill in the art to use a sequencing reaction to quantify donor-derived nucleic acids, much less diagnosing, predicting or monitoring a status or outcome of a transplant recipient based on the quantity of the sequenced donor-derived nucleic acids.

Accordingly, Applicant respectfully requests that this rejection of Claim 36 and dependent claims therefrom be withdrawn.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number STAN-706

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS
LLP

Date: _____

By: _____
Pamela J. Sherwood
Registration No. 36,677

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CAREDXVI00001858

A1005

	Application No.	Applicant(s)
Notice of Allowability	13/508,318	QUAKE ET AL.

	Examiner	Art Unit	AIA (First Inventor to File) Status
	AMY M. BUNKER	1639	No

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address--*
All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to _the communication filed on 14 January 2014_.

　　☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are _36,39,41,44-49,51,54,56,57,66,67 and 70_. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov .

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
　　Certified copies:
　　　a) ☐ All　　b) ☐ Some　*c) ☐ None of the:
　　　　1. ☐ Certified copies of the priority documents have been received.
　　　　2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
　　　　3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).
　　　* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.

　　☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.
　　Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)
1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .

5. ☒ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/MARIA LEAVITT/
Primary Examiner, Art Unit 1633

A1006

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Allowable Subject Matter

1.   This is in reply to papers filed on January 14, 2014. Claims 36, 39, 41, 44-51, 53, 54, 56, 57, 66 and 67 are now pending. Claim 36 has been amended by Applicant's amendment filed on 01-14-2014.

Therefore, claims 36, 39, 41, 44-51, 53, 54, 56, 57, 66 and 67 are under examination.

The examiner acknowledges receiving an executed Declaration under 37 C.F.R. § 1.132 signed by John F. Beausang on January 30, 2014 ("Beausang Decl."), and filed on January 31, 2014.

2.   Applicant's representative was contacted on January 28, 2014 to amend method claims 36, 41, and 51; add claim 70; and to cancel claims 50 and 53.

Authorization for the examiner's amendment was given in a telephone interview with Pamela Sherwood on January 30, 2014.

Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103, and 112.

3.   Accordingly, claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 are allowed.

Reasons for allowance

4.   The following is an examiner's statement of reasons for allowance:

A1007

By amendment to the claims, Applicant has persuaded the Examiner that the prior art of Moreira et al. (Clinical Chemistry, 2009, 55(11), 1958-1966); in view of Lo Yuk-Ming *et al.* (U.S. Patent Application No. 20050282185, published December 22, 2005); and further in view of Baxter-Lowe et al. (Clinical Chemistry, 2006, 52(4), 559-561) as evidenced by Applied Biosystems (ABI Prism® 7000 Sequence Detection System, Assays-on-Demand Gene Expression Products Protocol, 2003, 1-40), alone or in combination, do not teach or disclose a method for detecting transplant rejection, graft dysfunction, or organ failure, particularly, wherein the method comprises: obtaining a genotype of donor-specific polymorphisms or a genotype of subject-specific polymorphisms, or obtaining both a genotype of donor-specific polymorphisms and subject-specific polymorphisms to establish a polymorphism profile for detecting donor cell-free nucleic acids, wherein at least one single nucleotide polymorphism (SNP) is homozygous for the subject if the genotype comprises subject-specific polymorphisms comprising SNPs; multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile to detect donor cell-free nucleic acids and subject cell-free nucleic acids, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure, and wherein the sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).

The Examiner has also been persuaded by the Beausang Decl., indicating that the method of claim 36 would work with genotype information from the donor, genotype information from the transplant recipient or genotype information from both the donor and the transplant recipient, where the information from the genotype data can be used to determine which SNPs in the donor differ from the recipient by at least one allele, such that in the event data for only one genotype is available (either donor or recipient) the genotype of the other can be inferred from publically available databases (e.g.; dbSNP) that report the most likely allele frequencies in the population at each SNP site.

Examiner's Amendment

A1008

5.   In the claims:

Claims 50 and 53 have been cancelled.

Claim 70 has been added.

Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 have been rewritten as follows:

1-35.   (Cancelled)

36.   A method for detecting transplant rejection, graft dysfunction, or organ failure, the
       method comprising:
       (a)   providing a sample comprising cell-free nucleic acids from a subject who has
             received a transplant from a donor;
       (b)   obtaining a genotype of donor-specific polymorphisms or a genotype of
             subject-specific polymorphisms, or obtaining both a genotype of donor-specific
             polymorphisms and subject-specific polymorphisms, to establish a
             polymorphism profile for detecting donor cell-free nucleic acids, wherein at
             least one single nucleotide polymorphism (SNP) is homozygous for the subject
             if the genotype comprises subject-specific polymorphisms comprising SNPs;
       (c)   multiplex sequencing of the cell-free nucleic acids in the sample followed by
             analysis of the sequencing results using the polymorphism profile to detect
             donor cell-free nucleic acids and subject cell-free nucleic acids; and
       (d)   diagnosing, predicting, or monitoring a transplant status or outcome of the
             subject who has received the transplant by determining a quantity of the donor
             cell-free nucleic acids based on the detection of the donor cell-free nucleic
             acids and subject cell-free nucleic acids by the multiplexed sequencing,
             wherein an increase in the quantity of the donor cell-free nucleic acids over
             time is indicative of transplant rejection, graft dysfunction or organ failure, and

A1009

wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).

37.   (Cancelled)

38.   (Cancelled)

39.   The method of claim 36, wherein the polymorphism profile comprises one or more genetic variations selected from single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, insertion elements, insertions, repeats, or deletions.

40.   (Cancelled)

41.   The method of claim 36, wherein the cell-free nucleic acids are deoxyribonucleic acid (DNA).

42.   (Cancelled)

43.   (Cancelled)

44.   The method of claim 36, wherein the multiplexed sequencing comprises shotgun sequencing.

45.   The method of claim 36, wherein the multiplexed sequencing occurs in a single container.

46.   The method of claim 36, wherein the multiplexed sequencing comprises sequencing at least ten different nucleic acids.

A1010

47.   The method of claim 36 further comprising administering an immunosuppressive
       drug.

48.   The method of claim 36, wherein the diagnosing, predicting, or monitoring
       transplant status or outcome comprises treating a transplant rejection in a subject
       in need thereof.

49.   The method of claim 36, wherein the diagnosing, predicting, or monitoring
       transplant status or outcome comprises determining, modifying, or maintaining an
       immunosuppressive regimen.

50.   (Cancelled)

51.   The method of claim 36 further comprising conducting an assay selected from:
       digital polymerase chain reaction (PCR), real-time polymerase chain reaction
       (RT-PCR), array, or any combination thereof.

52.   (Cancelled)

53.   (Cancelled)

54.   The method of claim 36, wherein the polymorphism profile comprises at least one
       single nucleotide polymorphism.

55.   (Cancelled)

56.   The method of claim 36, wherein the sample is blood or serum.

57.   The method of claim 36, wherein the sample is urine or stool.

58-65.　(Cancelled)

66.　　The method of 36, wherein the transplant is selected from the group consisting of: kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

67.　　The method of claim 36, wherein the transplant is a heart transplant or kidney transplant.

68.　　(Cancelled)

69.　　(Cancelled)

70.　　The method of claim 36, wherein the cell-free nucleic acids are ribonucleic acid (RNA).

Claims 36, 39, 41, 44-49, 51, 54, 56, 57, 66, 67 and 70 are allowed.

Any inquiry considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Any inquiry concerning this communication or earlier communications from the examiner should be directed to AMY M. BUNKER whose telephone number is (313) 446-4833. The examiner can normally be reached on Monday through Friday 7:00am to 4:00pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Heather Calamita can be reached on (571) 272-2876. The fax phone number for the organization where this application or proceeding is assigned is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/AMY M BUNKER/
Examiner, Art Unit 1639

/MARIA LEAVITT/
Primary Examiner, Art Unit 1633

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application:	Confirmation No.: 6003
Inventor: Stephen R. Quake, et al.	Examiner: Amy M. Bunker
Application No.: 13/508,318	Group Art Unit: 1639
Filed: July 19, 2012	Customer No.: 77974
Title: **NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS**	

FILED ON: January 30, 2014

DECLARATION UNDER 37 C.F.R. § 1.132

Dear Madam:

I, JOHN F. BEAUSANG, declare as follows:

1. I am currently a Scientist II at ImmuMetrix, Inc.

2. From 2011-2013, I was a post-doctoral fellow in the laboratory of Stephen R. Quake at Stanford University, where I studied high throughput sequencing applications in genomics, cancer biology and immunology.

3. I am familiar with the prosecution history of the above-identified patent application.

4. I am submitting this declaration, and the attached Exhibit A, to demonstrate that genotype information obtained from a transplant recipient can be used to establish a polymorphism profile to detect donor-derived cell-free nucleic acids in a sample from the transplant recipient.

5. The attached Exhibit A is a figure showing an analysis that I performed showing the detection of donor-derived cell-free DNA based on genotype information obtained from a transplant recipient. The figure reflects an analysis that I performed on sequencing and genotyping data generated by others.

6. The underlying data for the figure was obtained from banked plasma samples from transplant recipients at Stanford University hospital. DNA from the samples was purified using commercially available kits. Sequencing libraries were constructed from cell-free DNA using

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commercially available kits and sequenced following standard protocols using an Illumina GAII sequencer. Genotypes of the transplant recipients were obtained from genomic DNA using the Illumina Omni1-Quad Beadchip following standard protocols. Data was analyzed using a combination of commercial, publicly available and custom computer code. For additional details see T.M. Snyder et al., 2011. Universal noninvasive detection of solid organ transplant rejection. *Proceedings of the National Academy of Sciences USA*, 108(15): 6229-6234.

7.   The attached Exhibit A shows that the time of peak donor-derived, cell-free DNA in the transplant recipient was 5 months. The time points shown in Exhibit A correspond to time points of when biopsies were performed on the transplant recipient. At 5 months, rejection was detected by biopsy and the transplant recipient was treated for transplant rejection (for additional details see T.M. Snyder et al., 2011. Universal noninvasive detection of solid organ transplant rejection. *Proceedings of the National Academy of Sciences USA*, 108(15): 6229-6234). Thus, Exhibit A shows that detection of donor nucleic acids based on genotype information from a transplant recipient can be used to diagnose, predict or monitor a transplant status or outcome.

8.   Based on the results described in Example 1 of the Specification and the results from the attached Exhibit A, I believe the method of claim 36 would work with genotype information from the donor, genotype information from the transplant recipient or genotype information from both the donor and the transplant recipient. Information from the genotype data can be used to determine which SNPs in the donor differ from the recipient by at least one allele. These sites can then be used to identify and tally donor-specific and recipient-specific sequencing reads from the cell-free DNA, and to observe the increase in donor DNA during rejection events. In the event that data for only one genotype is available (either the donor or the recipient) the genotype of the other can be inferred from publicly available databases (e.g., dbSNP) that report the most likely allele frequencies in the population at each SNP site. For example, if the recipient genotype at a particular SNP site is known to be AA, the database reports 50%A and 50%C, and the sequencing data from cell-free DNA reports a 'C' then, even if the donor genotype was not measured, it can be in inferred with high likelihood that this sequencing read can be attributed to the donor since it matches the database.

9.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

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statements were made with the knowledge that willful false statements and the like so made are
punishable by fine or imprisonment, or both, under 18 U.S.C. 1001.

Executed on this 30th day of January 2014.

JOHN F. BEAUSANG

Scientist II

ImmuMetrix, Inc.

3183 Porter Drive

Palo Alto, CA 94025

A1016

Exhibit A

Donor DNA (%)

Patient # 7
Recipient-Only Genotype

Grade 3A rejection event
determined by biopsy

Months Post-Transplant

A1017

Universal noninvasive detection of solid organ transplant rejection

Thomas M. Snyder[a,b], Kiran K. Khush[c], Hannah A. Valantine[c,1], and Stephen R. Quake[a,b,1]

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It is challenging to monitor the health of transplanted organs, particularly with respect to rejection by the host immune system. Because transplanted organs have genomes that are distinct from the recipient's genome, we used high throughput shotgun sequencing to develop a universal noninvasive approach to monitoring organ health. We analyzed cell-free DNA circulating in the blood of heart transplant recipients and observed significantly increased levels of cell-free DNA from the donor genome at times when an endomyocardial biopsy independently established the presence of acute cellular rejection in these heart transplant recipients. Our results demonstrate that cell-free DNA can be used to detect an organ-specific signature that correlates with rejection, and this measurement can be made on any combination of donor and recipient. This noninvasive test holds promise for replacing the endomyocardial biopsy in heart transplant recipients and may be applicable to other solid organ transplants.

next-generation sequencing | noninvasive diagnosis | acute rejection

The surveillance of organ health, particularly to detect the onset of transplant rejection, is essential for the long-term survival of organ transplant recipients. For heart transplant recipients, the gold standard for diagnosis of rejection is the endomyocardial biopsy. However, the endomyocardial biopsy is an expensive and invasive procedure that is limited by sampling error and interobserver variability in grading. Furthermore, cardiac biopsies may cause patient discomfort and rare but serious complications, including arterial puncture, arrhythmias, conduction abnormalities, biopsy-induced tricuspid regurgitation, and even cardiac perforation (1–5).

There has been considerable effort to develop noninvasive techniques that might replace or reduce the need for endomyocardial biopsies, with much focus placed on monitoring the recipient's immune response to detect the onset of rejection. The expression profile of certain genes in peripheral blood mononuclear cells (PBMCs), assayed from patient blood samples, has been demonstrated to differ between quiescent patients and those with severe rejection episodes (6–8). The AlloMap molecular expression test (XDx) is the first FDA-approved test based on this research (9, 10). This test has a low positive predictive value; however, its use in conjunction with clinical observation and echocardiograms has been shown to safely reduce the number of biopsies performed without increasing risk of serious cardiovascular events (9).

Instead of monitoring the recipient's immune response, we have developed an assay that directly interrogates the health of the donated organ. This technique involves measuring the signature of dying cells from the organ in the cell-free DNA circulating in the recipient's plasma (11). If a unique genomic signature of DNA from the donated organ (compared with the recipient's genome) can be identified, then the level of "donor DNA" from the transplanted organ can be monitored over time, and changes in organ health can be detected as changes in the donor DNA level. (Fig. 1) The rationale for this approach arises from the observation that both acute and chronic rejection processes are associated with apoptosis of specific cell types within the allograft (12, 13). Past research has attempted to identify cell-free DNA in sex-mismatched female recipients of male donor organs, where chromosome Y can serve as the donor genetic signature. This line of research, however, has yielded conflicting results on the existence of a donor-specific signature in the plasma of organ transplant recipients (14, 15). The clearest evidence has come from renal transplantation, where

donor-specific chromosome Y has been detected in recipient urine and plasma (16–19). To date, most measurements of cell-free DNA in organ transplantation have been limited to the special case of women who receive male organs, which has prevented the widespread use of cell-free DNA as a diagnostic tool, because female recipients of male donor organs represent less than a quarter of all transplant procedures. HLA markers can be quantified to identify donor-derived DNA in pancreas–kidney transplant recipients (20), but the precision is low, making its utility to measure rejection unclear, and it is not applicable to cases when the donor and recipient are HLA matched.

Here, we show that organ-specific donor DNA is detectable in the plasma of heart transplant recipients and that this genetic signature increases substantially before rejection events. We also demonstrate a universal, sex-independent strategy using shotgun sequencing to measure single nucleotide polymorphism (SNP) differences between individuals to quantify the donor DNA signal. This genome transplant dynamics (GTD) approach is applicable to any organ donor and any recipient, regardless of sex, by first genotyping the donor and recipient to establish a unique donor "genetic fingerprint," which can be detected by high throughput sequencing of cell-free DNA in the recipient's blood following transplantation. The GTD assay provides a quantitative measure of organ health that can complement or possibly replace other approaches for posttransplant monitoring.

Results

Chromosome Y Detection in Sex-Mismatched Transplant Recipients. Because previous research has reported conflicting results on the possibility of detecting genetic signatures from transplanted organs using conventional PCR (14, 15), we first explored the use of the more sensitive technique of microfluidic digital PCR (21, 22) in sex-mismatched transplants where a female recipient has received a male donor heart. We purified DNA from the plasma of nine patients collected immediately before an endomyocardial biopsy that established a cellular rejection episode ($\geq$grade 3A/2R). Six patients (identified as patients 1–6) were females who had received a heart from a female donor and three patients (identified as patients 7–9) were females who had received a heart from a male donor. Digital PCR was performed on the cell-free DNA using probes for chromosome (Chr) 1 and chromosome Y to establish the % Chr Y signal relative to Chr 1 (Fig. 2A). For the six female patients receiving organs from female donors, either no or a very low level of Chr Y was observed $0.32 \pm 0.27\%$ (SD). However, for the three female patients who received male organs, in four unique plasma samples (one patient had two documented rejection events), a greater than tenfold higher average level of Chr Y was observed,
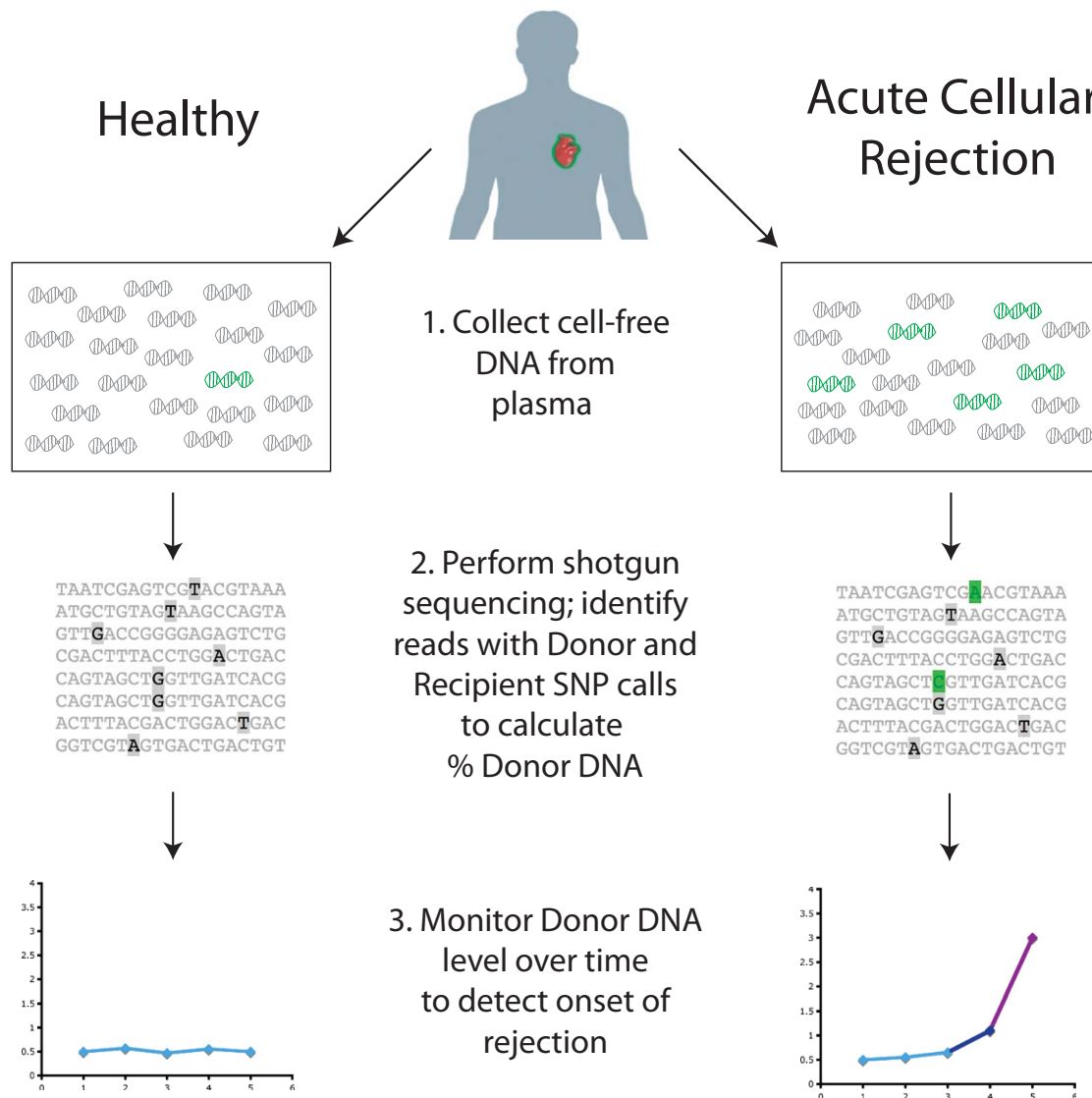
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Fig. 1. General scheme for this study. Cell-free DNA collected in plasma contains a majority of molecules from the recipient (in gray) but may also include some from the transplanted organ (green). Due to increased cell death in the organ during a rejection episode, more donor molecules are expected to be present in the blood at these times. Shotgun sequencing of the purified DNA allows for counting recipient versus donor molecules by looking at single nucleotide polymorphisms (SNPs) that vary between donor and recipient. Very high levels of donor DNA, particularly changes from past measurements, will indicate the onset of rejection.

$3.93 \pm 3.07\%$ (SD), with a range from 1.4 to 8.2%. The signal from the male-donor samples is well separated from that of the female-donor controls ($P = 0.018$, Student's t test). These data establish that genetic material from the transplanted organ can be detected in the recipient's plasma during rejection episodes.

We then expanded our analysis to a larger set of 39 archived samples from female transplant patients who had received male hearts, some of whom had rejection events and some of whom did not. Each patient had been sampled at multiple time points and an endomyocardial biopsy was performed after each blood draw. Example time series are shown in Fig. 2 and Fig. S1; one can clearly see that donor DNA levels from the heart are increased at rejection events. Using all samples from all time points, we plotted a receiver operating curve (ROC) on the basis of different thresholds for the donor DNA level, with the endomyocardial biopsy results (grades $\geq$3A–2R) used as the indicator of true positive rejection events

(Fig. S2). At a threshold of 2.0% donor DNA, we capture an 80% true positive rate with a 15% false positive rate. Our data suggest that values of donor DNA less than 1% and typically around 0.5% appear "normal" for heart transplant recipients; higher values are likely indicative of organ damage similar to that directly observed by endomyocardial biopsy showing myocyte damage that characterizes rejection grades $\geq$3A–2R, with a significant difference between the donor DNA levels in the five true positives and the other 34 samples analyzed ($P = 0.0002$, Student's t test).

For patient 10, there is a high level of chromosome Y at the first time point, 2 wk after transplantation. Examination of the clinical record revealed the presence of antibody-mediated rejection, due to presensitization before transplantation with high levels of circulating HLA antibodies directed against donor antigens. Following aggressive treatment for antibody-mediated rejection, the signal for this patient stabilized around $0.60 \pm 0.41\%$ (SD) for the

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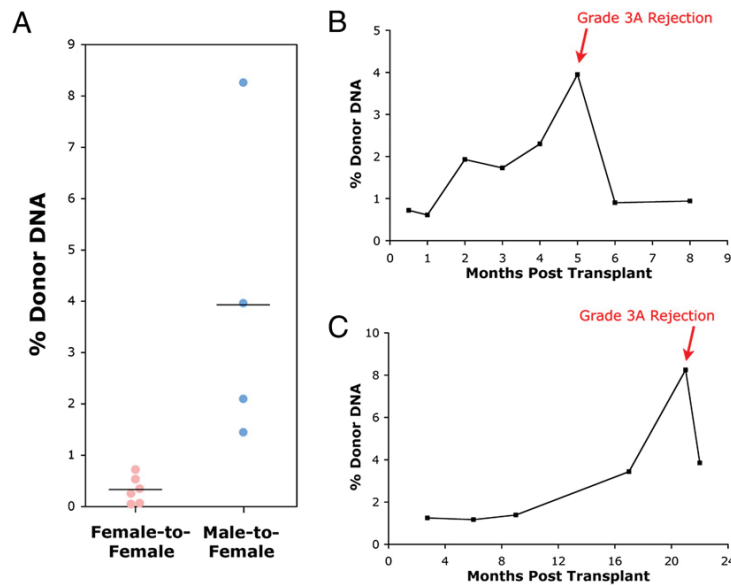
Fig. 2. Donor DNA levels determined by digital PCR using a chromosome Y marker. (*A*) Ten plasma samples from patients with biopsy-determined rejection events with grade ≥3A–2R were analyzed, including six female patients (1–6) receiving female organs (six total events) and three female patients (7–9) receiving male organs (four total events). Group averages are marked by black lines. (*B* and *C*) Time-course graphs of chromosome Y levels (% donor DNA) in patients 7 (*B*) and 8 (*C*). Both patients had grade 3A–2R rejections as determined by biopsy at the indicated time point.

other six plasma samples collected in the first year. This first data point is therefore treated as a true positive in our analysis. For patient 13, a high chromosome Y signal is seen both immediately following transplantation and at 8 mo after transplantation. These spikes in signal are not connected to any documented events in this patient's medical history, and in the absence of such records we treat them as false positives. For other patients without documented rejection events, there is no significant chromosome Y signal above 0.50% at any monitored time point (Fig. S1).

Samples after rejection episodes were also analyzed, where possible from the archived plasma record, to determine whether donor DNA levels decreased following treatment. For patient 7, samples both 1 and 3 mo following rejection were analyzed and revealed that the donor DNA levels had fallen significantly, to ~0.9%. For patient 8, only one sample, 1 mo after treatment began, was available and it also showed a reduction in donor DNA from its peak at the time of rejection, albeit to a value that is still higher than what was seen before the rejection event. Details on each patient's medical status at the time of rejection and following treatment are given in Table S1.

Sequencing-Based Donor DNA Quantitation. Although the above results demonstrate that rejection can be detected using chromosome Y as a genetic signature for the donor organ's DNA in the plasma, this type of assay can only be used in the minority of cases where a female recipient receives a male donor organ. We sought to demonstrate that using more detailed knowledge of the genomes of the donor and recipient—such as a large number of single nucleotide polymorphisms—could be used to monitor the genome transplant dynamics between arbitrary pairings of donor and recipient.

A frequent estimate for the variation between individuals is that approximately one base per thousand differs, for about 3 million total SNPs (23). However, not all of these sites will be useful for discriminating recipient and donor molecules in the plasma. Due to the overwhelming number of expected recipient DNA molecules in the plasma, the only usable locations are those where the recipient has a homozygous SNP with a single base present in both alleles. This leaves about 1.6 million positions to query by sequencing, of which about a quarter will be homozygous for a different allele in the donor's genome and the rest heterozygous with just one of the two bases differing.

For each usable SNP, we can identify the "recipient" base (i.e., A, from an AA SNP), the single base present in both alleles of that SNP in the recipient's genome. The donor base (i.e., T, from an AT or TT SNP) is the new base present in either one (heterozygous) or both (homozygous) alleles of the donor's genome that is not

present in the recipient's genome. Any other possible base calls at the site (i.e., C or G) would be considered an "error" from PCR amplification and/or sequencing. Whenever a shotgun-sequencing read aligns over a site containing an identified usable SNP, we assign the read to one of three bins, depending on whether it shows the recipient base, the donor base, or something else at the SNP site. Using the total count of recipient and donor bases, we then calculate the ratio of donor DNA present. Although we may not observe reads from any particular SNP, by having hundreds of thousands of potential loci to discriminate donor and recipient molecules, there should be enough reads to establish an accurate donor DNA percentage. A diagram of the workflow used to assign SNPs into groups and to assign DNA sequences to donor or recipient is given in Fig. S3.

As proof of principle, we took genetic material from two HapMap cell lines (NA07348 and NA10830) that have been heavily genotyped, and treated one cell line as recipient and the other as donor (23). Approximately 660,000 usable homozygous recipient SNPs are characterized in these cell lines, with 160,000 of these being homozygous donor SNPs and the rest heterozygous donor SNPs. We mixed genomic DNA from NA10830, the mock "donor," with DNA from NA07348, the mock recipient, at ratios between 1.5 and 7.5%, and prepared libraries for sequencing. A control library with just the recipient DNA—"0% donor"—was also created. Each library was sequenced in a single lane on an Illumina GAII, yielding between 3.6–10.8 million unique aligned sequences of which 30,000–100,000 contained SNP locations (Table 1). Recipient, donor, and error calls were counted from the sequenced bases. Whereas the majority of calls were from the recipient genome, as expected, an increasing number of donor calls was made as the proportion of donor DNA in the library increased. Illumina's quality scores were used to remove a majority of sequencing errors, and the number of recipient and donor calls was much larger than the overall sequencing error rate.

The raw counts were then used to calculate donor percentages, using just the homozygous SNPs, just the heterozygous SNPs, or total SNPs as shown in Fig. 3. Provided a correction is made for seeing only one out of every two donor molecules for the heterozygous SNPs, data from either heterozygous or homozygous donor SNPs give a reliable calculation of % donor DNA. The overall response was linear over the sampled range ($R^2 = 0.998$) with sufficient sensitivity to measure transplant rejection, on the basis of the digital PCR results for donor percentage in the actual patient samples. These results establish a methodology to use SNPs to quantify the amount of one genome present in the background of another.

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Table 1. Sequencing statistics for the control HapMap genomic libraries

% donor	0.0	1.5	2.0	2.5	4.0	5.0	7.5
Total reads	13,082,100	6,321,400	8,707,200	14,116,000	9,169,000	12,510,700	19,183,300
Aligned	9,019,118	4,581,260	6,629,726	10,885,018	6,278,041	7,552,199	14,145,769
Unique	8,747,074	3,651,817	5,145,156	7,076,860	5,077,904	5,003,458	10,824,332
Total reads with SNPs	77,201	30,421	46,892	63,884	45,708	39,047	98,384
Heterozygous SNPs							
Total reads	58,047	22,812	35,410	48,293	34,541	29,348	74,099
Recipient reads	57,852	22,549	34,841	47,329	33,587	28,310	70,268
Donor reads	138	244	533	925	928	1,009	3,803
Errors	57	19	36	39	26	29	28
Homozygous SNPs							
Total reads	19,154	7,609	11,482	15,591	11,167	9,699	24,285
Recipient reads	19,088	7,465	11,149	15,044	10,544	8,978	21,882
Donor reads	59	137	327	539	610	706	2,394
Errors	7	7	6	8	13	15	9

Reads from either heterozygous or homozygous donor SNPs are separated from the other calls. Only SNP base calls with a quality score (QS) $\geq$80 are used to minimize base-calling errors. The number of "error" calls is significantly less than the number of "donor" calls, even for the 0% donor library, which may result from errors in the established genotype with false homozygous calls. As only one in two reads for a heterozygous donor SNP will contain the donor base, the overall rate of such reads is about half the rate for homozygous donor SNPs.

SNP Analysis of Patient Plasma Samples by Sequencing. To demonstrate the feasibility of using SNPs as a marker for GTD in patient samples, we first needed to establish genotype information for the donor and recipient. We obtained whole blood from the recipient and banked splenocytes from the donor for patients 7 and 8, with rejection events, and patient 11, who had no such events. DNA was purified from these cells and was then genotyped at over a million loci using Illumina's Omni1-Quad Beadchip. Because our assay is particularly sensitive to false positive homozygous SNP calls for the recipient, we limited our focus to Beadchip SNPs with high GenCall and Cluster Separation scores, which yielded ~150,000 usable loci in each case. Improvements in genotyping technology should eventually allow for many more of the potential 1.6 million usable SNPs to be identified, thereby improving the GTD assay's sensitivity even further.

Sequencing libraries were prepared from the purified plasma DNA for each patient as previously described (24). Shotgun sequencing led to an average of 10–12 million unique aligning reads per sample, with ~25,000 SNP-containing reads per time point (complete statistics are given in Table S2). Total donor DNA % was calculated using all SNPs (homozygous and heterozygous) and the results analyzed for each time point in each patient as before. Example time course graphs are shown in Fig. 4 and show similar trends to those observed by digital PCR for these same patients.

Whereas our initial sequencing experiments were performed on samples from female recipients of male donor hearts to independently validate the results against the digital PCR measurements, we sought to demonstrate the universal nature of this approach by analyzing patients that could not be tracked using chromosome Y. We therefore performed our GTD assay on four male patients who received male organs (patients 14–17). A time course for one of these patients is shown in Fig. 4C.

The GTD assay allows for an internal control for the genetic signature of the donor organ compared with experimental background. In addition to considering all of the SNPs that differ between donor and recipient, the set of homozygous SNPs identified as the same for donor and recipient can be considered. Any non-recipient signal observed at these sites will represent the assay background arising from sequencing errors, genotyping errors, or potential sample contamination with other human genetic material that would vary at some of these positions. The error in matched SNPs is plotted in green for these patients in Fig. 4 and is stable over all time points. The rise seen during biopsy-proven rejections is only seen in the donor-specific SNPs and therefore must be a specific signal from the donor organ that does not arise from changes in sequencing error rates or from sample contamination. The distance between the two curves is a reasonable value to report for observed donor DNA. Whereas several months before rejection this difference is negligible, this difference can rise to over 3–4%, a significant level of donor DNA, at the biopsy-proven rejection time points.

We generated an ROC curve for the collected sequencing data of all 44 patient samples (Fig. S4). Using biopsy grades as the indicator of "true positives" (rejection events), at a threshold of 1.70% donor DNA we can capture an 83% true positive rate with a 16% false positive rate. Comparing the donor DNA levels for the 6 true positives (2.75 $\pm$ 1.81%, SD) to the 38 other samples (0.92 $\pm$ 1.16%, SD) reveals a significant difference between the rejection samples and the other analyzed time points ($P = 0.0013$, Student's t test).

Although we have treated all time points in this study that were not coincident with a biopsy-proven rejection event as "negatives," the observed trends by digital PCR and sequencing suggest that some of the time points, particularly those immediately before the rejection, may be elevated above baseline as an early indication of the onset of graft damage. For our sequencing results, we grouped together all time points not within 3 mo of an observed acute cellular rejection as "healthy." Remaining time points were either grouped together as close to rejection or at rejection. The collected data from these groups are shown in Fig. 4D. Whereas some samples in the intermediate group have low values similar to the healthy time points, many have intermediate or high values similar to the rejection time points, suggesting that in some cases it may be possible to use this assay for earlier detection of rejection. Although more patient samples, particularly with biopsies graded at intermediate levels, will be needed to establish the significance of time points immediately before
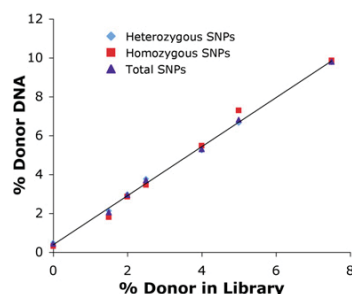

Fig. 3. SNP-based detection of % donor DNA in control HapMap samples. There is a linear response ($R^2 = 0.998$) of calculated % donor DNA compared with the % donor in the mock sequencing libraries. The trendline is given for all SNPs, including both heterozygous and homozygous donor SNPs, but the calculated percentages are similar in both subsets.
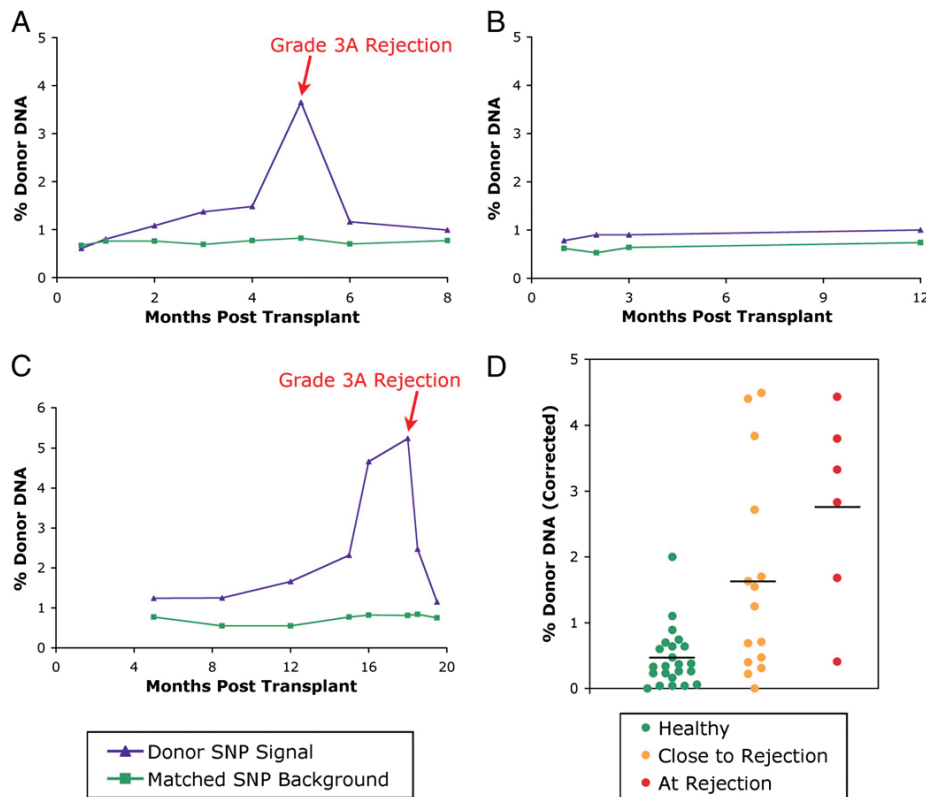
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Fig. 4. Donor DNA levels determined by sequencing. (*A–C*) Time-course graphs for patients 7 (*A*) and 11 (*B*), female patients receiving male hearts also analyzed by digital PCR, and for patient 14 (*C*), a male patient receiving a male heart. Patients 7 and 14 had grade 3A–2R rejections as determined by biopsy at the indicated time point, whereas patient 11 was a negative control with no rejection events. The calculated donor SNP signal is graphed in purple using both homozygous and heterozygous donor SNP positions. The matched SNP background is graphed in green, demonstrating the error for the assay arising from sequencing errors, genotyping errors, or sample contamination. The difference between the green and purple trend lines represents the changing level of donor-specific DNA. (*D*) Collected sequencing results for all patients analyzed by sequencing (7, 8, 11, 14–17) using the corrected % donor DNA values. Samples coincident with a biopsy determining an acute cellular rejection event are grouped together in red. Samples within 3 mo of a biopsy-determined rejection are shown in orange. Samples greater than 3 mo from any biopsy-determined rejection, or from a patient without any rejection events, are considered "healthy" normal readings and are shown in green. Group averages are marked by black lines.

biopsy-determined rejections, our results clearly establish significant differences from normal once graft damage is severe, as determined by a grade ≥3A–2R biopsy.

Discussion

In this study we aimed to demonstrate that donor-derived cell-free DNA exists in the plasma of organ transplant recipients, and that elevated levels of donor DNA can be used as an indication of organ rejection. Although the existing cell-free DNA literature has presented conflicting reports on whether organ-specific signatures can be detected in plasma (14, 15), our data establish unambiguously that donor-specific DNA is present in the plasma of heart transplant recipients. By both methods of GTD demonstrated here, we establish a mean value below 1% as being normal for the level of donor-derived cell-free DNA when the patient is healthy. During organ rejection, however, the level of donor DNA signal rises in correlation with the endomyocardial biopsy results, with mean values increasing to 3–4% of the total cell-free DNA. Following treatment, the level of donor DNA tends to decline, and in several patients returns to baseline. Collectively these results establish that donor-derived DNA in the plasma is a promising biomarker for the onset of, and recovery from, heart transplant rejection. Whereas most earlier studies focused on the limited cases of females receiving male organs, here we have also demonstrated a generalizable strategy using single nucleotide polymorphisms that can be used for any possible donor and recipient pair.

In comparing GTD to noninvasive expression analysis tests, such as AlloMap, one observes some similarities and differences. It is not known whether AlloMap can detect rejection before biopsy, whereas we have shown evidence here that GTD is able to detect rejection before biopsy. Neither GTD nor AlloMap have been shown to distinguish between antibody-mediated rejection and cellular rejection. However, both conditions are treated with corticosteroids while awaiting a confirmatory test, so the fact that GTD can perform early

detection may enable early intervention to prevent full blown rejection, whether cellular or antibody mediated. Because GTD measures the genetic signature of the donor organ, it should, like the endomyocardial biopsy, more directly report organ damage.

What could the health economic benefits of GTD be? Although it is difficult to calculate the precise value of early detection, there have been calculations of the benefit of using a noninvasive test to reduce the number of biopsies. On the basis of the results of the CARGO study, Evans et al. estimated that a noninvasive test with similar performance properties could save $12 million annually in health care costs in the United States (25). Because the GTD false positive rate is about half of that of the AlloMap test, the savings would be even greater, not including the benefits of early detection. Further studies, particularly of GTD in the clinic, will be required to determine the complete utility of this test as a replacement for the biopsy.

Because GTD and the AlloMap test look at different signals in the blood, and likely have different sources for false positives/ negatives, a combination of the two approaches could be particularly powerful by reporting on both host immune response and graft injury. As GTD is not particularly dependent on physiology specific to the heart, it also has the potential to be used in the setting of other solid organ transplants (such as kidney, lung, and liver), where DNA from the transplanted organ may also exist in the recipient's plasma.

Materials and Methods

Posttransplant Monitoring and Clinical Sample Collection. This study used stored plasma samples from a previously established cohort of 112 consecutive patients undergoing first heart transplantation between January 2002 and May 2005 at our institution. This cohort, funded by the National Institutes of Health (5P01AI050153-02), was assembled prospectively to study the relationship between cytomegalovirus (CMV) infection and the development of cardiac allograft vasculopathy. Age younger than 10 y, renal dysfunction requiring prolonged dialysis, and inability or unwillingness to provide signed

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informed consent represented exclusion criteria for study enrollment. All patients gave informed consent to the protocol approved by our institutional review board for studies in human subjects.

Posttransplant immunosuppression consisted of daclizumab (1 mg/kg i.v.) administered at the time of transplant surgery and on alternate weeks for a total of five doses, cyclosporine (3–5 mg/kg/d); prednisone initiated at 1 mg/kg/d and tapered to <0.1 mg/kg/d by the sixth postoperative month; and either mycophenolate mofetil 1,000–4,000 mg daily, or sirolimus 1–4 mg daily. All recipients received standard CMV prophylaxis consisting of 4 wk of i.v. ganciclovir. Those recipients who were CMV antibody negative and received a heart from a CMV antibody positive donor received an additional 3 mo course of CMV hyperimmune serum and up to 80 d of valganciclovir.

All study patients were monitored for acute cellular rejection by surveillance endomyocardial biopsies performed at scheduled intervals after transplant: weekly during the first month, biweekly until the third month, monthly until the sixth month, and then at months 9 and 12. Biopsies were graded according to the 1990 International Society for Heart and Lung Transplantation (ISHLT) classification system as 0, 1A, 1B, 2, 3A, 3B, and 4 (26). These grades are readily translatable to the ISHLT 2004 revised grading scale (0, 1R, 2R, and 3R) (27). Plasma samples were collected before performing the biopsy procedure and stored at the following time points posttransplant: day 14 and months 1–4, 6, 9, 12, 16, 20, 24, 38, 32, 36, 40, 44, 48, 52, 56, and 60.

Stored plasma samples were used for this study as follows: Serial plasma samples were retrieved for 13 patients with at least one episode of biopsy-proven acute cellular rejection (≥grade 3A–2R). Six of these 13 patients were females who had received hearts from female donors, 3 were females who had received hearts from male donors, and 4 were from males who had received hearts from male donors. Plasma was also retrieved for 4 female patients receiving hearts from male donors with no rejection episodes (all biopsies grade 0, or 1A–0, or 1R). For the recipients with male donors, plasma samples from as many as eight different time points, including any biopsy-proven rejection time points, were analyzed to determine a time course for the donor-specific DNA signature.

Plasma Purification and Digital PCR. Plasma samples (1–2 mL total volume) were purified using the Nucleospin Plasma F kit (E&K Scientific Products). Digital PCR was performed on 12.765 digital array chips using the BioMark real-time PCR system (Fluidigm); FastStart TaqMan Probe Master Mix with Rox (Roche) was used with two probes targeted to a Chr 1 locus (EIF2C1) and a multicopy Chr Y locus (Dys14), as previously described (24). Control male and female genomic DNA (Promega) was used to calibrate the Chr 1 and Chr Y signals.

Control SNP Library Preparation. Genomic DNA for the NA07348 and NA10830 HapMap lines was attained from the Coriell Institute for Medical Research (Camden, NJ). DNA was quantitated using the NanoDrop spectrophotometer (Thermo Scientific) and mixed at defined ratios. DNA was sheared to ~200 to 300-bp fragments on a Covaris S2 (ABI) and purified on a Microcon YM-30 column (Millipore) before performing Illumina's single-end sequencing library preparation.

Patient Genotyping and Library Preparation. Recipient DNA from whole blood and donor DNA from banked splenocytes was purified using the DNeasy blood and tissue kit (Qiagen). Where necessary, DNA was amplified by Repli-G Midi kit (Qiagen) before sending the samples to SA Biosciences for genotyping on the Omni1-Quad Beadchip (Illumina). To minimize false positive homozygous recipient SNP calls, only SNP loci with a GenCall score ≥0.70 and a Cluster Separation score of 1.00 were considered.

Sequencing libraries were prepared from the purified patient plasma DNA using the standard Illumina library preparation method with the exception of reduced adaptor concentration during ligation as previously described (24).

Sequencing. Thirty-six–cycle single-end sequencing runs were performed for all DNA libraries. Each library was analyzed on a single lane of an Illumina GAII flowcell, with the exception of the second sample from patient 8, which combined data from two lanes due to poor sequencing yields. Reads were aligned to the reference human genome hg18 using ELAND, with an average of over 13 million aligned reads per lane and over 7 million unique aligning reads per lane. As nonunique reads at this low level of coverage most likely arise from the PCR amplification during library preparation, duplicated reads (reads that aligned to the same location) were removed before analysis to leave just a single aligning read at each site. All unique reads that span one of the SNP sites where the recipient has a homozygous allele that differs from the donor's genotype were then analyzed for the presence of a recipient, donor, or error (other) base call. Bases with reported quality scores lower than 80 were excluded from this analysis to minimize sequencing errors. The total donor DNA percentage was calculated by taking twice the number of donor heterozygous read calls plus the number of donor homozygous read calls over the total number of donor and recipient read calls, not including errors.

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C1010

A1023

Ashbridge, Beth

From:	Dwyer, Anna <Anna.Dwyer@weil.com>
Sent:	Wednesday, January 27, 2021 3:56 PM
To:	DeJong, Kevin J
Cc:	Jefferson, Julius; bfarnan@farnanlaw.com; Reines, Edward; mfarnan@farnanlaw.com; CareDx Natera; Woo, Darryl M; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor
Subject:	RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Kevin,

Thanks for your edits, these are acceptable to CareDx. We will provide final versions prior to filing. In the future, please provide redline versions of any edits Eurofins provides to joint submissions so we can discern them promptly and efficiently.

Additionally, we disagree with Eurofins' characterization of yesterday's meet-and-confer. As CareDx explained, the disputed "sensitivity" limitation is clear on its face, and therefore not indefinite.

Best,
Anna

From: DeJong, Kevin J <KDeJong@goodwinlaw.com>
Sent: Wednesday, January 27, 2021 2:46 PM
To: Dwyer, Anna <Anna.Dwyer@weil.com>
Cc: Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>; Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Anna,

Thanks for the meet and confer yesterday, and for preparing the drafts. As you know, we had asked CareDx to articulate the plain and ordinary meaning of the disputed "sensitivity" limitation, and CareDx was not able to provide a construction. Accordingly, our position remains that the term is indefinite.

Please see attached for our edits to the cover pleading and Exhibit B. Exhibit A looks fine. Please send us the final versions before filing today.

Thanks,

Kevin

From: Dwyer, Anna <Anna.Dwyer@weil.com>
Sent: Tuesday, January 26, 2021 3:13 PM
To: DeJong, Kevin J <KDeJong@goodwinlaw.com>
Cc: Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>; Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-

1

A1024

CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Counsel,

As discussed, please find CareDx's draft joint claim construction chart materials. Please revert back to us once the chart in Exhibit B has been populated with Eurofins' intrinsic evidence so we can prepare for tomorrow's filing.

Best,
Anna

From: DeJong, Kevin J <KDeJong@goodwinlaw.com>
Sent: Tuesday, January 26, 2021 1:04 PM
To: Dwyer, Anna <Anna.Dwyer@weil.com>
Cc: Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>; Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: Re: CareDx/Eurofins- CareDx Proposed Claim Construction

Anna- We can do 3 pm EST. Same call-in number.

Sent from my iPhone

On Jan 26, 2021, at 12:57 PM, Dwyer, Anna <Anna.Dwyer@weil.com> wrote:

Hi Kevin,

I am no longer available to meet and confer at 5:30 ET today. Please provide an alternative time prior to 3:30 PM.

Best,
Anna

From: DeJong, Kevin J <KDeJong@goodwinlaw.com>
Sent: Tuesday, January 26, 2021 12:49 PM
To: Dwyer, Anna <Anna.Dwyer@weil.com>; Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>
Cc: Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Anna,

We can do 530 pm EST today. Let's use this call-in number:

1-844-302-9816; Passcode 5892746339
Mobile-friendly: 18443029816,,5892746339#

2
A1025

Thanks,

Kevin

From: Dwyer, Anna <Anna.Dwyer@weil.com>
Sent: Monday, January 25, 2021 2:28 PM
To: DeJong, Kevin J <KDeJong@goodwinlaw.com>; Jefferson, Julius <JJefferson@goodwinlaw.com>;
bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com;
CareDx Natera <CareDx.Natera@weil.com>
Cc: Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor
<DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Kevin – apologies, to clarify, we are not available from 3:30-5 PM ET. We are available any other time
tomorrow afternoon.

Best,
Anna

From: Dwyer, Anna <Anna.Dwyer@weil.com>
Sent: Monday, January 25, 2021 2:22 PM
To: DeJong, Kevin J <KDeJong@goodwinlaw.com>; Jefferson, Julius <JJefferson@goodwinlaw.com>;
bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com;
CareDx Natera <CareDx.Natera@weil.com>
Cc: Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor
<DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Kevin,

We are available tomorrow afternoon between 3:30-5 PM ET for a meet-and-confer. Please circulate a
dial-in. Below please find CareDx and Stanford's proposed constructions for the terms that Eurofins
Viracor identified.

'652 Patent Claim Term	CareDx/Stanford's Proposed Construction
"A method for detecting . . . the method comprising: (a) providing a sample . . . (b) obtaining a genotype . . . (c) multiplex sequencing . . . (d) diagnosing, predicting, or monitoring . . . ."	Steps 1(a) – 1(d) are not required to be completed in the recited order.
"diagnosing, predicting, or monitoring a transplant status or outcome"	The term "diagnosing, predicting, or monitoring a transplant status or outcome" includes "predicting or diagnosing the transplant status or outcome, determining predisposition to a transplant status or outcome, monitoring treatment of transplant patient, diagnosing a therapeutic response of transplant patient, and prognosis of transplant status or outcome, transplant progression, and response to particular treatment."
"wherein sensitivity of the method is greater than 56% compared to sensitivity of current	Not indefinite, plain and ordinary meaning

| surveillance methods for cardiac allograft vasculopathy (CAV)" | |

Best,
Anna

From: DeJong, Kevin J <KDeJong@goodwinlaw.com>
Sent: Monday, January 25, 2021 11:02 AM
To: Dwyer, Anna <Anna.Dwyer@weil.com>; Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>
Cc: Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: RE: CareDx/Eurofins- CareDx Proposed Claim Construction

Anna,

The parties are to meet and confer to prepare a joint claim construction chart to be filed this Wednesday, June 27. We are available tomorrow afternoon EST to confer. In order to have a productive meet and confer, please provide us today with CareDx and Stanford's proposed constructions for the terms that Eurofins Viracor identified.

Best,

Kevin

From: Dwyer, Anna <Anna.Dwyer@weil.com>
Sent: Thursday, January 21, 2021 6:00 PM
To: Jefferson, Julius <JJefferson@goodwinlaw.com>; bfarnan@farnanlaw.com; DeJong, Kevin J <KDeJong@goodwinlaw.com>; Reines, Edward <edward.reines@weil.com>; mfarnan@farnanlaw.com; CareDx Natera <CareDx.Natera@weil.com>
Cc: Woo, Darryl M <DWoo@goodwinlaw.com>; kkeller@shawkeller.com; DG-CareDX v Eurofins Viracor <DG-CareDXvEurofinsViracor@goodwinlaw.com>
Subject: CareDx/Eurofins- CareDx Proposed Claim Construction

Counsel,

Pursuant to the amended scheduling order, CareDx does not propose any claim terms of the '652 patent for construction.

Regards,
Anna

A1028

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on _10 October 2013_.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
2a)☒ This action is **FINAL.**   2b)☐ This action is non-final.
3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) _36,39,41,42,44-51,53,54,56,57,66 and 67_ is/are pending in the application.
   5a) Of the above claim(s) _____ is/are withdrawn from consideration.
6)☐ Claim(s) _____ is/are allowed.
7)☒ Claim(s) _36,39,41,42,44-51,53,54,56,57,66 and 67_ is/are rejected.
8)☐ Claim(s) _____ is/are objected to.
9)☐ Claim(s) _____ are subject to restriction and/or election requirement.
* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.
11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
   Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
   Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   Certified copies:
   a)☐ All   b)☐ Some * c)☐ None of the:
      1.☐ Certified copies of the priority documents have been received.
      2.☐ Certified copies of the priority documents have been received in Application No. _____.
      3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
   * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
4) ☐ Other: _____.

CAREDXVI00001842

A1029

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office Action.

Status of Claims

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are currently pending. Claims 36, 42, 44, 50 and 51 have been amended by Applicants amendment filed on 10-10-2013. Claims 40, 43 and 58 have been canceled by Applicant's amendment filed on 10-10-2013.

Applicant's election *with traverse* of Group I, claims 36, 39-51, 53, 54, 56-58, 66 and 67, with traverse and the election of species: (A) single nucleotide polymorphisms (B) kidney transplant in the reply filed on March 21, 2013 was previously acknowledged.

No claims were withdrawn from consideration.

A complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

Accordingly, claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are under consideration to which the following grounds of rejection are applicable.

Priority

The present application is a 35 U.S.C. 371 national stage filing of International Application No. PCT/US2010/055604, filed on November 5, 2010, which claims the benefit of US Patent Application No. 61/280,674, filed on November 6, 2009.

Interview Summary

The telephone interview between the Examiner, Primary Examiner Maria Leavitt, Pamela Sherwood, Kim Stopak and Lucia Muntean on August 16, 2013 discussing the scope of the obviousness rejection of record was previously acknowledged.

Withdrawn Rejections

Applicants' amendment and arguments filed April 16, 2013 are acknowledged and have been fully considered. The Examiner has re-weighed all the evidence of record. Any rejection and/or objection not specifically addressed below are herein withdrawn.

Claim Rejections - 35 USC § 112

The rejection of claims 36, 39-51, 53, 54, 56, 57, 66 and 67 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn, and the rejection of claim 58 is rendered moot, for the recitation of the term "derived from" due to Applicants amendment of claim 36 to replace the term with "wherein the one or more nucleic acids originated from the transplant donor" and Applicants cancellation of claim 58.

The rejection of claims 36, 39-51, 53, 54, 56, 57, 66 and 67 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn, and the rejection of claim 58 is rendered moot, for insufficient antecedent basis for the term "transplant status" due to Applicants amendment of claim 36 to recite proper antecedent basis and Applicants cancellation of claim 58.

Claim Rejections - 35 USC § 103

The rejection of claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are under 35

U.S.C. 103(a) is withdrawn, and the rejection of claims 40, 43 and 58 are rendered moot, as

being unpatentable over Lo Yuk-Ming *et al.* (U.S. Patent Application No. 20050282185,

published December 22, 2005) in view of Saint-Mezard *et al.* (International Patent Application

No. WO2009060035A1, published May 14, 2009) due to Applicants amendment of the claims

and Applicants cancellation of claims 40, 43 and 58.

The combined references of Lo Yuk-Ming et al. and Saint-Mezard et al. have been
narrowed by reciting, "detecting the one or more circulating, cell-free nucleic acids from the
transplant of the donor" vs. "detecting one or more nucleic acids derived from the transplant
from the donor (previous claim 36) which detecting comprises sequencing (claim 43, now
canceled), wherein the sensitivity of the method is 56%.In view of the withdrawn rejection,
applicant's arguments are rendered moot.

New Objections/Rejections

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all

obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or
> described as set forth in section 102 of this title, if the differences between the subject
> matter sought to be patented and the prior art are such that the subject matter as a whole
> would have been obvious at the time the invention was made to a person having ordinary
> skill in the art to which said subject matter pertains. Patentability shall not be negatived
> by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the

claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various

claims was commonly owned at the time any inventions covered therein were made absent any

evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out

the inventor and invention dates of each claim that was not commonly owned at the time a later

invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c)

and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are rejected under 35 U.S.C.

103(a) as being obvious over Moreira et al. (Clinical Chemistry, 2009, 55(11), 1958-1966); in

view of Lo Yuk-Ming *et al.* (U.S. Patent Application No. 20050282185, published December 22,

2005); and further in view of Baxter-Lowe et al. (Clinical Chemistry, 2006, 52(4), 559-561) as

evidenced by Applied Biosystems (ABI Prism® 7000 Sequence Detection System, Assays-on-

Demand Gene Expression Products Protocol, 2003, 1-40). **This is a new rejection necessitated**

by amendment of the claims in the response filed October 10, 2013.

Moreira et al. teach the use of total cell-free DNA (t-CF-DNA) and **donor-derived cell-**

free DNA (ddCF-DNA) from **urine** and **plasma** as a rapid non-invasive **biomarker** of **rejection**

and long-term graft function and survival in **renal transplant patients** (kidney transplant)

(instant claims 36, 41, 56, 57, 66 and 67) (pg. 1958, column 1, Background, entire paragraph),

such that plasma and urine samples from 100 renal **transplant recipients** were obtained 3

months after transplantation (providing a sample from a subject who has received a transplant)

and tCF-DNA and ddCF-DNA were analyzed by **quantitative PCR** (multiplex reaction/real-

time PCR) of the HBB and TSPY1 genes using a Prism® 7000 Sequence Detection System by

Applied Biosystems (shotgun sequencing), where plasma tCF-DNA concentrations increased

markedly during acute rejection (AR) episodes, often before clinical diagnosis, and returned to

reference values after **anti-rejection treatment** (administering an immunosuppressive drug)

(instant claims 36, 42, 44, 47, 48 and 51) (pg. 1958, column 1, Methods, entire paragraph &

column 1, Results, lines 1-4). Moreira et al. also teach that a cut-off plasma of 12,000 genome

equivalents/mL tCF-DNA concentration correctly classified AR and non-AR episodes in 86% of

post-transplantation complications (diagnostic sensitivity, 89% and specificity, 85%) (sensitivity

greater than 56%) (instant claim 36) (pg. 1958, column 1, Results, lines 4-8). Moreira et al.

further teach that ddCF-DNA concentrations, up to 2000 genome equivalents/mL (detect at least

ten different nucleic acids), were measured in women who had received a graft from a male

donor and that ddCF-DNA was detected immediately after transplantation in patients without AR

and infection, while plasma concentrations were undetectable within the first week after

transplantation, such that patients who developed AR and graft infection showed a marked

increase in the concentration of ddCF-DNA, which became undetectable after appropriate

immunosuppressive treatment (pg. 1958, columns 1 & 2, Utility of ddCF-DNA, entire paragraph and pg. 1963, Fig 4.).

Moreira et al. do not teach where the one or more nucleic acids derived from the donor are detected based on a marker profile comprising one or more genetic variations selected from those listed in instant claim 39 (instant claim 39), or where detecting comprises at least ten different nucleic acids (instant claim 46); or where the transplant status or outcome comprises modifying or maintaining an immunosuppressive regimen (instant claim 49); or where detecting comprises detecting genetic variations (instant claim 50); or where the method of claim 50 comprises genetic variations listed in instant claim 53 (instant claim 53); or where the method comprises at least one single nucleotide polymorphism (instant claim 54). Although Moreira et al. do not specifically teach where the multiplexed reaction occurs in a single container, the Prism® 7000 Sequence Detection System is designed with plates holding individual wells (single container) as evidenced by Applied Biosystems (pg. 5) (instant claim 45).

The Lo Yuk-Ming et al. reference is relied upon for the reasons of record. Particularly, Lo Yuk-Ming et al. teach a method of differentiating DNA of an organ **donor** from **DNA** of an organ **recipient** using a **biological sample** such as plasma or serum (e.g., cell-free DNA) in order to **predict** the clinical progress of the transplantation recipient especially applied to **organ rejection** (paragraph [0030]), where DNA includes any sequence of more than one nucleotide such as polynucleotides, gene fragments and complete gene sequences, as well as, the study of **single nucleotide polymorphisms** (SNPs) (elected species), as well as, methylated and unmethylated alleles (e.g., genetic variations) (instant claims 39, 50, 53 and 54) (paragraphs [0012], lines 6-8, [0019] and Fig. 2). Lo Yuk-Ming et al. teach that the assay is applicable to the study of cellular chimerism following **solid organ** transplantation (e.g., liver, spleen, heart, pancreas, and kidneys), post-translational plasma DNA chimerism and urinary DNA chimerism. Lo Yuk-Ming et al. also teach SNPs in SEQ ID NOS: 1-11 (detecting at least ten different nucleic acid sequences) (instant claim 46) (pgs. 8-10, sequence listing).

The combined references of Moreira et al. and Lo Yuk-Ming et al. do not teach where the transplant status or outcome comprises modifying or maintaining an immunosuppressive regimen (instant claim 49).

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Baxter-Lowe et al. teach that one of the most promising areas of transplantation research is the discovery of biomarkers for rejection that are detectable in blood and urine, such that the development of non-invasive assays detecting molecular biomarkers for rejection by; (a) detecting a pre-rejection profile that will allow therapeutic interventions before rejection causes graft dysfunction, (b) improving the sensitivity and specificity of rejection diagnosis, (c) developing new classification systems for rejection that improve prognosis, and (d) providing information for designing individualized **immunosuppressive regimes** that could prevent rejection while minimizing drug toxicity (determining/modifying immunosuppressive regimen) (instant claim 49) (pg. 559, first full paragraph).

In view of the teachings of Moreira et al., which exemplifies the use of total cell-free DNA (tCF-DNA) and donor-derived cell-free DNA (ddCF-DNA) from urine and plasma as a highly sensitive, rapid and non-invasive biomarker of rejection and long-term graft function and survival in renal transplant patients, where tCF-DNA and ddCF-DNA were analyzed by quantitative PCR; and in view of the teachings of Lo Yuk-Ming et al., which disclose a method of differentiating DNA of an organ donor from DNA of an organ recipient using a biological sample such as plasma or serum in order to predict the clinical progress of the transplantation recipient especially applied to solid organ rejection, where DNA includes any sequence of more than one nucleotide such as polynucleotides, gene fragments and complete gene sequences, as well as, the study of single nucleotide polymorphisms (SNPs), as well as, methylated and unmethylated alleles (e.g., genetic variations); and in view of the teachings of Baxter-Lowe et al., which describe the discovery of biomarkers for rejection that are detectable in blood and urine, such that the development of non-invasive assays detecting molecular biomarkers for rejection by detecting a pre-rejection profile that will allow therapeutic interventions before rejection causes graft dysfunction and providing information for designing individualized immunosuppressive regimes that could prevent rejection while minimizing drug toxicity; one of ordinary skill in the art at the time the invention was made would be motivated to use the variants taught by Lo Yuk-Ming et al. in the method taught by Moreira et al. to analyze donor-derived cell free DNA for the diagnosis and prognosis of graft rejection and, furthermore, to apply a pre-rejection profile for the development therapeutic intervention regimens as taught by Baxter-Lowe et al. for an efficient and non-invasive early detection of biomarkers of organ

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rejection and the development of personalized immunosuppressive regimens. In addition, one of ordinary skill in the art would have a reasonable expectation of success in using the genetic variants taught by Lo Yuk-Ming et al. in the method taught by Moreira et al. to detect biomarkers in urine and blood without having to conduct invasive biopsies in order to diagnosis transplant rejection and to allow for early immunosuppressive intervention as taught by Baxter-Lowe et al. This reasonable expectation of success would motivate one of ordinary skill in the art to modify the cited references.

The cited references teach the limitations of the claims and, therefore, the invention, as a whole, was *prima facie* obvious to one of ordinary skill in the art.

Conclusion

Claims 36, 39, 41, 42, 44-51, 53, 54, 56, 57, 66 and 67 are rejected.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to AMY M. BUNKER whose telephone number is (313) 446-4833. The examiner can normally be reached on 7:00am - 4:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Heather Calamita, can be reached on (571) 272-2876. The fax phone number for the organization where this application or proceeding is assigned is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

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The Role of Echocardiography in the Follow-Up of Orthotopic Heart Transplant Patients

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Echocardiography is being used increasingly in the follow-up of patients after orthotopic heart transplantation. Doppler echocardiographic parameters of diastolic function (isovolumic relaxation time (IVRT), pressure half time (PHT), and early peak mitral inflow velocity (MI)) have been shown to be useful in detecting moderate to severe allograft rejection. Using each individual patient as his/her own control, accounting for the effects of recipient atrial contraction and time from transplant, a decrease in the IVRT or PHT tx 15% is considered diagnostic of diastolic dysfunction and hence, is an echocardiographic diagnosis of rejection. The sensitivity of these parameters to predict rejection approaches 80–90% with a specificity of 80–90%. Echocardiography is the procedure of choice in the diagnosis and follow-up of pericardial effusion and tricuspid regurgitation after transplantation. The presence of tricuspid regurgitation seems to be related to and a complication of the number of endomyocardial biopsies performed during the first year after transplantation. Dobutamine stress echocardiography is being used increasingly in the noninvasive diagnosis of transplant coronary artery disease. Using angiography as the "gold standard," dobutamine stress echocardiography has a sensitivity of 80–96% in the detection of transplant coronary artery disease. Echocardiographic measures of both systolic and diastolic function, and the development of regional wall motion abnormalities during dobutamine stress echocardiography, have all been shown to be useful predictors of mortality and the occurrence of cardiac events late after transplantation. (Cardiol Rev 1996;4:6, 297–307)

Key words: heart transplantation, echocardiography, stress echocardiography, allograft function, Doppler, rejection, diastolic dysfunction, pericardial effusion, tricuspid regurgitation, cardiac transplantation

The current "state of the art" for detection of allograft rejection after orthotopic heart transplantation relies on the use of serial surveillance endomyocardial biopsies. The validation of a noninvasive tool to detect rejection would decrease complications related to biopsy, radiation exposure, and cost by obviating the need for repetitive invasive procedures. However, to date, all proposed noninvasive modalities for monitoring rejection have been hampered by inadequate sensitivity and specificity. The purpose of this paper is to review the current status of the role of echocardiography (M-mode, two-dimensional, and Doppler) in following patients after transplantation, with specific emphasis on the role of echocardiography in the diagnosis of allograft rejection.

DOPPLER ECHOCARDIOGRAPHY IN THE DIAGNOSIS OF REJECTION

Acute rejection is characterized histologically by myocardial mononuclear cell infiltration and edema in the perivascular space and interstitial myocardium (1). These changes lead to increased stiffness of the myocardium, alterations in left ventricular filling properties, and diastolic function that can be measured by Doppler echocardiography. Doppler echocardiographic indices during rejection are consistent with a restrictive physiology; specifically, an increase in early mitral inflow velocity (MI) (measured vertically from the baseline to peak early mitral inflow velocity), a decrease in the isovolumic relaxation time

isovolumic relaxation time (IVRT) (time from aortic valve closure to mitral valve opening), and a decrease in the pressure half time (PHT) (the time required for the peak left atrial (LA)/left ventricular (LV) pressure difference to decline by one-half). These indices have been shown to correlate with the presence of cellular rejection on endomyocardial biopsy after orthotopic heart transplantation (2–7) (Table 1).

TECHNICAL POINTERS

Patients should be studied in the left lateral decubitus position after 15 minutes of recumbency to verify basal heart rate and blood pressure. Blood flow across the mitral valve is measured using pulsed wave Doppler with the transducer positioned at the cardiac apex. Mitral velocities are recorded from a four-chamber view, with the sample volume located at the mitral valve leaflet tips. In this position, the discrete aortic valve closure signal is also recorded. All subsequent Doppler echo recordings of mitral flow velocity and aortic and mitral valve movements are performed with the sample volume in the same position. It is imperative that the angle between blood flow and ultrasonic beam be as close as possible to zero. Small deviations result in large discrepancies in the velocity profile; specifically, angles $\geq 20°$ result in serious underestimation of the velocity profile. Three important technical factors must be addressed to adequately assess the information from Doppler echocardiography studies for diagnosis of rejection:

- The residual recipient atrial remnant usually maintains electrical and mechanical function. When recipient atrial contraction occurs during the donor's late systole, it increases LA pressure, leading to earlier mitral valve opening and shortening of the IVRT. When the mitral valve opens, the higher LV diastolic pressure results in a more rapid decline in the LA/LV pressure difference, therefore shortening the PHT and increasing the MI. This gives a false impression of restrictive physiology consistent with rejection. Conversely, if the recipient atrial contraction occurs in late diastole (simultaneously with donor atrial contraction), this results in an augmented A wave and increased LV filling at end-diastole, a pattern termed *relaxation abnormality*. To minimize the influence of recipient atrial contraction on the early diastolic mitral inflow pattern, only beats in which recipient atrial contraction occurs in diastole and early systole should be used for analysis of IVRT, MI and PHT (8, 9). The mean value for each parameter should be calculated from 10 such acceptable consecutive cycles.
- It must be recognized that the characteristic or "normal" postoperative Doppler echocardiography mitral inflow pattern very early after transplantation is consistent with restrictive physiology. This diastolic abnormality, previously reported in experimental studies, evolves slowly over the ensuing 6 weeks to a less restrictive pattern (16),

as the heart recovers from ischemia. Thus, Doppler echocardiography parameters early after transplantation tend to be restrictive in pattern, reflecting injury caused by ischemia rather than rejection. However, failure to recover normal diastolic function (an increase in IVRT and PHT and a decrease in MI) in the first 6 weeks after transplantation is consistent with a Doppler echocardiographic definition of rejection. This pattern emphasizes the importance of serial studies, with each patient acting as his/her own control, particularly early after transplantation.

- Because of wide individual variability, each individual patient's previous Doppler diastolic indices must be used for reference (17). Interindividual variation precludes the use of a standardized value of IVRT, PHT, or MI that can be used to define diastolic dysfunction and, hence, rejection. Based upon studies assessing the spontaneous interpatient variability of Doppler echocardiography measures of diastolic function, a decrease in the IVRT or PHT of $> 15\%$ from a patients previous study represents a significant change, diagnostic of diastolic dysfunction. The IVRT and PHT are rarely discordant. However, discordance might be seen when the IVRT prolongs because of early aortic valve closure as a consequence of decreased systolic function and LV stroke volume. In this event, an increase in the MI of 20% from previous studies is considered diagnostic of diastolic dysfunction (4). The presence of ongoing diastolic dysfunction on consecutive studies is also considered diagnostic of restrictive physiology consistent with rejection.

> Because of wide individual variability, each individual patient's previous Doppler diastolic indices must be used for reference.

Table 1 displays the sensitivities and specificities that have been calculated in several studies assessing the role of Doppler echocardiography in diagnosis of cellular rejection. The sensitivities of IVRT and PHT have ranged from 60 to 88%, although the majority of studies show a sensitivity in 80–88% range. The specificities have ranged from 57 to 90% but (again) are predominantly in the 80–90% range. The majority of these studies did account for the timing of recipient atrial contraction and used a 15–20% decrease in IVRT or PHT as diagnostic of moderate to severe rejection. Two earlier studies that found Doppler echocardiography unhelpful in diagnosing rejec-

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TABLE 1
Sensitivity and specificity of studies assessing Doppler diastolic function in the diagnosis of rejection after transplantation

Study	Patients (n)	EMB correlates (n)	Sensitivity (%)	Specificity (%)	Technical notes	Comments
Valantine et al. (4)	22	120	88		did not adjust for RAC	15 decr IVRT—myocyte necrosis
Valantine et al. (3)	39	114	76 82	79	noted RAC, each indiv. own control	15% decr in PHT 15 decr IVRT or PHT—moderate to severe rej
Desruennes et al. (5)	55		88	87	noted RAC	20% decr PHT
Dawkins et al. (7)	20		60	87		20% decr IVRT
Furniss et al. (12)	12	100	87	90	did not assess RAC	10% decr IVRT IVRT—2-year study correlation moderate to severe rej
Stork et al. (13)	21	21	82	80	noted RAC not longitudinal—2 examinations per pt group 1 no rej, group 2 developed rej	significant decr in IVRT (≥ 12 ms) and PHT during moderate to severe acute rej
Revista Cardiol (14)	56	163	85	57		IVRT: moderate acute rej
Amende et al. (15)	35	84			did not assess RAC	significant decr. in IVRT with rej

EMB, endomyocardial biopsy; RAC, recipient atrial contraction; decr, decrease; IVRT, isovolumic relaxation time; PHT, pressure half time; rej, rejection; pt, patient.

tion did not account for the effects of recipient atrial contraction (Table 2) (10, 11).

The use of diastolic filling parameters to routinely assess rejection remains limited for two reasons:

• Diastolic dysfunction can occur in the absence of cellular rejection on endomyocardial biopsy. In some patients, Doppler echocardiography dysfunction can precede the development of acute cellular rejection. Of 12 false-positive Doppler echocardiographic examinations in the study by Valantine et al. (3), moderate acute cellular rejection developed in 7 (58%) within 28 days. Lymphocytes present in small numbers on biopsy thought not to represent rejection can produce soluble mediators, such as cytokines, that lead to the abnormalities of diastolic function seen at Doppler examination. Both cytokines and markers of activated cytotoxic T cells, specifically granzyme A expression, have been associated with diastolic dysfunction assessed by Doppler echocardiography (18).

• Doppler echocardiography has a poor sensitivity in correlating with mild rejection on biopsy. However, in the absence of allograft dysfunction, the significance of mild cellular rejection on biopsy is unclear and it is not treated at most centers.

Because of the risk of endomyocardial biopsies in infants, many pediatric transplant centers base rejection surveillance primarily on echocardiographic and clinical parameters of LV function. In this setting, endomyocardial biopsy is performed when indicated by these noninvasive surveillance methods (19).

ECHOCARDIOGRAPHIC SCORING SYSTEMS USING MULTIPLE PARAMETERS

In an attempt to improve sensitivity and specificity, Ciliberto et al. (20) assessed the reliability of a multiparametric echocardiographic assessment in the noninvasive diagnosis of acute rejection in 1400 biopsy-correlated examinations in 130 patients. Echocardiographic parameters assessed included the following: *a*) diastolic function (20 millisecond decrease in IVRT or PHT, accounting for the effects of recipient atrial contraction), *b*) > 10% decrease in ejection fraction, *c*) an increase in myocardial echogenicity, *d*) appearance or increase in pericardial effusion, or *e*) an increase in wall thickness > 4 millimeters (interventricular septum plus left ventricular posterior wall). Each individual patient acted as his/her own control. The echocardiographic scoring system had a very high level of specificity (range 98.3–99.8%) with an overall sensitivity of 80% for predicting histologically moderate rejection. All false-negative, moderate rejection episodes were focal and resolved within 1 week. Echocardiographic criteria were also useful in predicting the patients' course

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TABLE 2
Negative studies regarding Doppler diastolic function in the diagnosis of rejection

Study	Patients (n)	# EMB	Measurement	Result	Problem
Forster et al. (10)	39	144	velocity HT	no difference	did not account for effects of RAC (10 consecutive beats) No IVRT measurement
Mannaerts et al. (11)	32	347	IVRT, velocity HT and deceleration time by pulsed wave Doppler	no difference	Did not account for effects of RAC ?? indiv pt as own control; grouped patients according to rejection levels
Spes et al. (47)	31	84	MI, PHT, %FS	no correlation	assessed use of DE in detection of mild rejection, no IVRT, accounted for RAC

EMB, endomyocardial biopsy; IVRT, isovolumic relaxation time; HT, half time; MI, early mitral inflow velocity; RAC, recipient atrial contraction; DE, Doppler echocardiography; FS, fractional shortening; pt, patient.

after treatment. Patients with a benign course had improvement in their echocardiographic picture, and patients with an unfavorable course had further worsening echocardiographically. Dodd et al. (21) combined a series of echocardiographic parameters into an echo score which, when applied prospectively, had a negative predictive value of 97%, ie. the likelihood that a patient would have a negative score with moderate/severe rejection on biopsy was extremely low.

M-MODE ASSESSMENT OF LEFT VENTRICULAR HYPERTROPHY

Before the introduction of cyclosporine, changes in left ventricular wall thickness and left ventricular mass as assessed by M mode echocardiography often accompanied acute cellular rejection (22, 23). However, the relationship between changes in wall thickness, left ventricular mass, and rejection in the cyclosporine era are less well delineated. Mannaerts et al. (24) correlated changes in 263 consecutive M-mode echocardiographic studies (left ventricular wall thickness, internal dimension and fractional shortening) with endomyocardial biopsies in 30 patients after heart transplantation. There was no significant correlation observed between the ultrasound variables and biopsy class and, therefore, no benefit in using M-mode to predict rejection. Recently, left ventricular hypertrophy was correlated with humoral rejection in a retrospective study in 14 patients early after transplantation (25); results that need confirmation in larger prospective studies.

OTHER ECHOCARDIOGRAPHIC MARKERS USED IN THE ASSESSMENT OF REJECTION

Park et al. (26) prospectively assessed early diastolic left ventricular function (Te, the time interval between maxi-

mal posterior wall contraction and the point of peak posterior wall endocardium retraction velocity) as a marker of acute cardiac rejection. In patients without rejection (48), Te was prolonged compared with healthy controls. During acute rejection, all 18 patients studied had significantly longer mean Te values compared with those without rejection. Longitudinal studies in 18 individual patients showed that rejection was associated with prolongation of Te, which returned to individual baseline values in response to treatment. When a 20% increase in Te was used prospectively in 96 patients, the sensitivity was 80% and specificity 94% in predicting acute rejection.

Simmonds et al. (27) assessed Doppler examination of superior vena caval flow in the detection of acute rejection. Thirty patients (15 with rejection) were studied within 36 hours of biopsy. Superior vena caval, mitral, and tricuspid early peak flow velocities were assessed. In the 15 patients with acute rejection, mitral and tricuspid flow velocities developed a restrictive type pattern, and superior vena caval flow was markedly abnormal, with an almost complete loss of forward systolic flow. If rejection was defined as a forward superior vena caval systolic flow $\leq$ 17 cm/sec, then the sensitivity was 100%, specificity 80%, and predictive accuracy 90%. This study did not look at the longitudinal utility of superior vena caval flow to predict rejection.

CONCLUSIONS REGARDING ECHOCARDIOGRAPHY AND DIAGNOSIS OF REJECTION

For a new diagnostic technique to be considered acceptable when compared with the "gold standard," a sensitivity and specificity of greater than 90% is needed. However, it must be remembered that Doppler echocardiography is being compared with a "gold standard" in endomyocardial

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biopsies that has considerable limitations. Definition of rejection by histopathologic criteria remains controversial (28), and endomyocardial biopsy sampling errors, inadequate tissue samples, and underestimation of the severity of autopsy-proven rejection may occur (29).

Doppler echocardiographic assessment of diastolic function approaches the 90% sensitivity and specificity required for noninvasive diagnosis of moderate to severe rejection. The significance of mild rejection, often not detected by Doppler echocardiography, in the absence of clinical symptoms or allograft dysfunction, is uncertain, and the finding is not treated at most centers. Therefore, a technique that correlates with rejection necessitating intensification of therapy may prove to be more useful clinically than one that correlates strictly with histology.

ECHOCARDIOGRAPHIC MARKERS OF OUTCOME AFTER TRANSPLANTATION

The use of echocardiography to predict outcome after transplantation has recently been assessed (30). A study performed at our center in 83 patients used a diastolic dysfunction score derived for each patient, defined as the number of studies with Doppler echocardiographic evidence of diastolic dysfunction divided by the total number of studies performed in the first 6 months. In this study, patients with greater diastolic dysfunction within 6 months after transplantation had a reduced late-term actuarial survival. Although the number of patients who died or were retransplanted in this study were small, the mean diastolic dysfunction score in these patients was significantly elevated when compared with patients who survived. The causes of death in these patients suggested that significant diastolic dysfunction may be a marker for early vascular injury that predisposed patients to the development of transplant coronary artery disease and support the importance of close follow-up and surveillance of patients with pronounced diastolic dysfunction early after transplantation.

Yeoh et al. (31) found that in the presence of mild rejection, associated echocardiographic or clinical allograft dysfunction, predicted a higher likelihood of progression to moderate rejection and occurred sooner after transplantation than mild rejection occurring without allograft dysfunction. The presence of systolic dysfunction (ejection fraction < 35%) in association with grade 3A or higher rejection has also been associated with an 80% first-year mortality (32).

The role of echocardiographic assessment of left ventricular function as a prognostic tool after transplant requires further prospective study. However, these preliminary studies support earlier follow-up and more aggressive therapy in patients who have pronounced diastolic or systolic dysfunction after transplantation.

STRESS ECHOCARDIOGRAPHY IN DIAGNOSIS OF TRANSPLANT CORONARY ARTERY DISEASE

Transplant coronary artery disease is a major limiting factor in the long-term outcome of patients after transplantation, often leading to death or retransplantation. Currently, most centers advocate the use of annual angiographic examinations to detect transplant coronary artery disease, as it is often clinically silent, presenting in late stages with heart failure, myocardial infarction or cardiac death. Coronary angiography is an invasive, expensive procedure and often compromises renal function that might already be tenuous. Transplant coronary artery disease generally involves epicardial vessels but is typically a diffuse disease that may be grossly underestimated by coronary angiography. Therefore, there would be many advantages to a noninvasive, highly sensitive, and less expensive method to detect transplant coronary artery disease. A positive result would lead to specific patient selection for angiography rather than subjecting all patients to it. Stress echocardiography [exercise and pharmacologic (dobutamine and dipyridamole)] has been shown to be sensitive and specific in the diagnosis of coronary artery disease in the nontransplant patient and, therefore, its ability to detect transplant coronary artery disease has recently been assessed (Table 3).

> Transplant coronary artery disease is a major limiting factor in the long-term outcome of patients after transplantation, often leading to death or retransplantation.

Akosah et al. (33) studied the sensitivity and specificity of dobutamine stress echocardiography in predicting angiographic transplant coronary artery disease in 41 patients using a 16-segment, 1–4 grade wall motion protocol (mean dobutamine dose 36 ± 11). Of 21 patients with abnormal angiograms, 20 had positive dobutamine stress echocardiographic tests (sensitivity 95%). There were 9 false-positive studies (specificity 55%) that might reflect the inability of angiography to detect microvascular transplant coronary artery disease (34) reflected as wall motion abnormality, rather than represent true false-positive studies. Of the 12 negative dobutamine stress echocardiography, angiography was negative in 11 (negative predictive value 92%); therefore, a negative dobutamine stress echocardiograph virtually rules out transplant coronary artery disease. Only

TABLE 3

Sensitivity and specificity of various studies assessing the role of dobutamine stress echocardiography in the detection of transplant coronary artery disease

Study	Patients (n)	Mean dobutamine dose (patients with TxCAD)	Echocardiographic assessment	Sensitivity (%)	Specificity (%)	Notes
Akosah et al. (37)	82	31 ± 2	16 segment; grade 1–4	96	53	only 45 patients took part in sensitivity and specificity
Herregods et al. (36)	28	24 ± 9	16 segment; grade 1–4			unable to identify angiographic TxCAD
Akosah et al. (33)	41	36 ± 1.1	16 segment; grade 1–4	95	55	Negative predictive accuracy 92%
Derumeaux et al. (35)	41	31 ± 8	16 segment; grade 1–4	86	91	37 of 41 patients included

TxCAD, transplant coronary artery disease.

length of time after transplantation independently predicted positive findings on angiography or dobutamine stress echocardiography. A cohort of 76 patients was then followed for 1 year to determine the prognostic value of dobutamine stress echocardiography in predicting the occurrence of major cardiac events. Over the following 12 months, none of the patients with normal dobutamine stress echocardiography had major cardiac events, whereas 12 patients with abnormal dobutamine stress echocardiography had 15 major events [acute onset heart failure (10), unstable angina (2), and cardiac death (3)], including two patients with normal coronary angiograms. The wall motion score index was higher in patients with cardiac events than in patients with positive dobutamine stress echocardiography without major cardiac events, suggesting a prognostic role for dobutamine stress echocardiography.

Derumeaux et al. (35) studied 41 patients (mean 40 ± 20 months after transplantation) who had dobutamine stress echocardiography (mean dobutamine dose 29–31 μg/kg/min) within 1 month of angiography. The overall sensitivity of dobutamine stress echocardiography was 86% and specificity was 91% in predicting angiographic coronary artery disease. At follow-up, 2 of 37 patients had myocardial infarctions, and both of these patients had abnormal dobutamine stress echocardiography. In contrast, Herregods et al. (36) also assessed dobutamine stress echocardiography after heart transplantation and found it was of low sensitivity and specificity. The discrepancies between the study of Herregods et al. and previous studies in transplant coronary artery disease detection may relate to the low mean dose of dobutamine used (24 μg/kg/min.), the time lag from angiography to dobutamine stress echocardiography (within 6 months), and the small numbers of patients analyzed (n = 28).

Akosah et al. (37) evaluated 82 patients with dobutamine stress echocardiography to determine whether dobutamine-induced wall motion abnormality was associated with angina and to characterize the clinical differences between patients with (n = 11) and without (n = 71) stress-induced angina. Of the 11 patients with stress-induced angina, all had regional wall motion abnormality on dobutamine stress echocardiography. Nine patients had coronary angiographic data available; significant disease was present in 8 (epicardial disease in 7), the location of which matched the wall motion abnormality. Patients with angina were later post-transplant, had higher wall motion abnormality, and required less dobutamine to induce an endpoint at dobutamine stress echocardiography.

The sensitivity of dipyridamole stress echocardiography was assessed by Ciliberto et al. (38) in 80 patients who underwent dipyridamole echocardiography within 48 hours of angiography. All 55 patients with normal coronaries had negative dipyridamole echocardiograms (specificity 100%). Of 25 patients with angiographic transplant coronary artery disease, 8 had positive dipyridamole echocardiograms (sensitivity 32%), 7 of whom had angiographic lesions > 50%. These investigators concluded that dipyridamole echo was not useful overall in screening because of a low sensitivity, but abnormalities during dipyridamole were highly specific and sensitive in the detection of stenosis > 50%. There were no cardiac events in patients with negative studies. Of the 25 patients with transplant coronary artery disease, 7 patients had cardiac events (follow-up 9.8 ± 4.5 months), all of whom showed wall motion abnormality at rest and 4 of whom showed wall motion abnormality after dipyridamole infusion.

Collings et al. (39) assessed the usefulness of stress echocardiography in diagnosis of transplant coronary heart disease in 51 consecutive patients with 55 stress echocardiography-angiographic data sets. Patients were not excluded if they had concurrent rejection. Stress echocardiography correctly excluded the presence of angiographic transplant coronary heart disease in 86% of patients but was associated with a high false-negative rate for detection of moderate coronary stenosis. Exercise stress echocardiography might have limited usefulness because of a low chronotropic response to exercise, a factor that does not

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